EFFECT OF DIETARY VITAMIN E AND LIPIDS ON SOME IMMUNE PARAMETERS OF TURBOT (*SCOPHTHALMUS MAXIMUS* L.)

by

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ABSTRACT

The effect of dietary vitamin E and dietary lipids on growth and immune parameters of juvenile turbot (*Scophthalmus maximus*) were investigated in a series of experiments. The aims of the studies were to maximise immune function through dietary modulation to counteract stress induced immunodepression resulting from high stocking densities.

In the first experimental trial, the vitamin E requirement for an optimum immune response was studied and revealed that vitamin E depletion induced poor health status, lower growth with some mortalities occurring at the end of the trial. However, supplementation of the diets with high levels of α-tocopherol although ensuring better growth did not significantly enhance most of the parameters measured at the end of the trial. The second trial aimed to test regimes coupling fresh or oxidised oil and low or high vitamin E supplementation. The results showed that vitamin E had a role in preventing peroxidation as high vitamin E supplementation improved some of the immunological parameters measured compared to fish fed with the same oxidised oil but low levels of vitamin E. By contrast low levels of vitamin E did not induce pathological conditions in fish fed with fresh oil showing the importance of dietary lipid in the evaluation of vitamin E requirements. Following this investigation another feeding trial was designed to look at the interaction of polyunsaturated fatty acids (PUFAs) and α-tocopherol on the immune parameters of juvenile turbot. Although liver lipid composition was affected by the diets and growth was enhanced by high vitamin E levels and a high ratio of (n-3)/(n-6) PUFAs no significant differences could be attributed to the lipid quality in the immunological parameters measured. Vitamin E supplementation enhanced the proliferation of kidney leucocytes when stimulated with lipopolysaccharide.

These studies give some information on the requirements for vitamin E and lipid quality of juvenile turbot.

All experimental work was carried out under the project home office licence number 30431 1

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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List of publications and conference contributions

- **M. Crampe**, S.R. Farley, A. Langston, and A.L. Pulsford, 1997. Measurement of phagocytosis: a comparative evaluation of microscopic and microplate methods. A paper presented at the Methodology in Fish Diseases conference, 24-26 September 1996, Aberdeen, Scotland.

- **M. Crampe**, A.L. Pulsford and S.J. Davies, 1997. The effect of dietary vitamin E and oil quality on the immune sytem of turbot (*Scophthalmus maximus*). A paper presented at the Vetrapharm fish conference, Inverness, Scotland, 13th of November 1996.

- A.L. Pulsford, **M. Crampe**, A. Langston, and P.J. Glynn, 1995. Modulatory effect of disease, stress, copper, TBT and vitamin E on the immune system of flatfish. Fish & Shellfish Immunology, No. 5, pp. 631-643.

- C. Thomas, **M. Crampe**, B. Evans, and A.L. Pulsford, (submitted). Application of assays for immune competence in Dab *Limanda limanda* to sea-going conditions. Aquatic Toxicology.

Reports submitted to industrial partners:

- The effect of vitamin E and vitamin C on immune system of turbot (*Scophthalmus maximus*). Provisional report-July 1994. Hoffman -La Roche, Basel, Switzerland, 34 pp.

- The effect of vitamin E on immune system of turbot (*Scophthalmus maximus*). December 1994. Hoffman -La Roche, Basel, Switzerland, 34 pp.

- The effect of vitamin E and oxidised oil on immune sytem of turbot (*Scophthalmus maximus*). May 1996. Hoffman -La Roche, Basel, Switzerland, 42 pp.

- The effect of vitamin E and polyunsaturated fatty acids on immune system of turbot (*Scophthalmus maximus*). November 1997. Hoffman-La Roche, Basel, Switzerland, 55 pp.

Signed .....

Date 22/03/99

LIST OF ABBREVIATIONS

AA : ..... Arachidonic acid

AB : ..... Antibody

AMP : ..... Adenosine monophosphate

ATP : ..... Adenosine triphosphate

AVMN : ..... 2, 2' azobis (2,4-dimethyl-valeronitrile)

BHT : ..... Butylated hydroxytoluene

cm^3 : ..... centimeter cube

CFU : ..... Colony-forming unit

CoA : ..... Coenzyme A

ConA : ..... Concanavalin A

CO_2 : ..... Carbon dioxide

COX : ..... Cyclooxygenase

CSFs : ..... Colony stimulating factors

C_{18} : ..... 18 carbon fatty acids

C_{20} : ..... 20 carbon fatty acids

C_{22} : ..... 22 carbon fatty acids

DAG : ..... Diacylglycerol

DHA : ..... Docosahexaenoic acid

DHGLA : ..... Di-homo gamma linoleic acid

DMSO : ..... Dimethyl sulfoxide

DTH : ..... Delayed type hypersensitivity

EFA : ..... Essential fatty acids

EPA : ..... Eicosapentaenoic acid

FA : ..... Fatty acid

FACS : ..... Fluorescence activated cell sorter

FAD : ..... Flavin adenine dinucleotide

$FADH_2$: ..... reduced form of FAD

FBS : ..... Foetal bovine serum

FCR : ..... Feed conversion ratio

FFA : ..... Free fatty acids

FITC : ..... Fluorescein isothiocyanate

g : ..... grams

g : ..... gravitational force, 9.81m/s

GALT : ..... Gut associated lymphoid tissue

GC : ..... Gas chromatography

GDP : ..... Guanosine diphosphate

GLA : ..... Gamma linolenic acid

GSH : ..... Glutathione

GSSG : ..... Oxidised glutathione

GTP : ..... Guanosine triphosphate

HA : ..... Haemagglutinin

HCl : ..... Hydrochloric acid

HDL : ..... High density lipoprotein

HETEs : ..... Hydroxyeicosatetraenoic acid

12-HETE : ..... 12-hydroxyeicosatetraenoic acid

HEWL : ..... Hen egg white lysozyme

HMG-CoA : ..... 3-hydroxy-3 methylglutaryl-CoA

HpETE : ..... Hydroperoxy eicosatetraenoic acid

HPLC : ..... High performance liquid chromatography

hr : ..... Hour

HRBC : ..... Human red blood cells

HSCoA : ..... Coenzyme A

H_2O : ..... Water

H_2O_2 : ..... Hydrogen peroxyde

HUFAs : ..... Highly unsaturated fatty acids

IFNγ : ..... Interferon gamma

Ig : ..... Immunoglobulin

IHNV : ..... Infectious haematopoietic necrosis virus

IL-1β : ..... Interleukin 1 beta

IL-2 : ..... Interleukin 2

IL-6 : ..... Interleukin 6

IP_3 : ..... Inositol triphosphate

IU : ..... International units

Kcal : ..... Kilocalorie

KCl : ..... Potassium chloride

kg : ..... Kilograms

$KHCO_3$: ..... Potassium bicarbonate

KOH : ..... Potassium hydroxide

l: ..... Litres

LDL : ..... Low density lipoprotein

L-15 : ..... Leibovitz L-15 tissue culture medium

LO : ..... Lipoxygenase

LPS : ..... Lipopolysaccharide

LT : ..... Leukotriene

LTA_4 : ..... Leukotriene A_4

LTB_4 : ..... Leukotriene B_4

LTB_5 : ..... Leukotriene B_5

LX : ..... Lipoxins

LXA_4 : ..... Lipoxin A_4

M : ..... Molar

MDA : ..... Malondialdehyde

mg : ..... milligrams

MIF : ..... Macrophage migration inhibitory factor

min : ..... minutes

mm : ..... millimetres

mM : ..... millimolar

MTS : ..... 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NAD^+ : ..... Nicotinamide adenine dinucleotide

NADH : ..... Reduced form of NAD

NaOH : ..... Sodium hydroxide

NBT : ..... Nitroblue tetrazolium

NDGA : ..... Nordihydroguaiaretic acid

NH_4 : ..... Ammonia

NO_2 : ..... Nitrites

NO_3 : ..... Nitrates

OD : ..... Optical density

OOHETE : ..... Hydroperoxy-eicosatetraenoic acids

PBMC : ..... Peripheral blood mononuclear cells

PBS : ..... Phosphate buffered saline

PFC : ..... Plaque forming cell

PG : ..... Prostaglandin

PGE_2 : ..... Prostaglandin E_2

$PGF_{1\alpha}$: ..... Prostaglandin $F_{1\alpha}$

PGH_2 : ..... Prostaglandin endoperoxides

PGI_2 : ..... Prostacyclin

PHA : ..... Phytohaemagglutinin

PIP_2 : ..... Phosphatidylinositol 4,5 biphosphate

PMA : ..... Phorbol myristate acetate

PMN : ..... Polymorphonuclear cells

PMS : ..... Phenazine methosulfate

PUFAs : ..... Polyunsaturated fatty acids

PWM : ..... Pokeweed mitogen

r : ..... Correlation coefficient

RBC : ..... Red blood cells

SE : ..... Standard error of the mean

SGR : ..... Specific growth rate

SI : ..... Stimulation index

SOD : ..... Superoxide dismutase

SPB : ..... Sodium phosphate buffer

SRBC :	Sheep red blood cells
TfR :	Transferrin receptor
TLC :	Thin layer chromatography
TNF-α :	Tumour necrosis factor alpha
TX :	Thromboxane
U :	Unit
VLDL :	Very low density lipoprotein
Vol :	Volume
W/V :	Weight/volume
Log :	Logarithm to the base 10
Log_e :	logarithm to the base e
µg :	microgram
µl :	microlitre
µm :	micrometre
°C :	Degree centigrade
Ø :	diameter
Σ :	sum

This PhD is dedicated to my parents and my sister
for their love and support throughout my life

CHAPTER 1 - INTRODUCTION

1.1 - FISH IMMUNE SYSTEM AND TURBOT (*Scophthalmus maximus*)

1.1.1 - Immune system of fish

The immune system of fish has been the subject of a recent book edited by Iwama and Nakanishi (1996) collecting chapters from several authors reviewing the different aspects of fish immune system; therefore the following section far from giving an extensive knowledge on fish immunity will only give an outline.

Jawed fish are phylogenetically the first group of animals to possess an acquired immune system characterised by the presence of lymphocytes, immunological memory and lymphoid tissue such as the thymus and spleen. It can be compared to the mammalian immune sytem although it has not been studied as extensively as the latter. A major difference between the two systems however is the fact that the fish immune system can be regulated by temperature (Avtalion, 1981). Teleosts are one of the largest and the most successful groups of modern fish with an estimated number of 23,000 species exceding by far the diversity of any vertebrate group (Beck *et al.*, 1991). Like mammals, teleosts possess various kinds of white blood cells ranging from monocytes and macrophages to different types of granulocytes, thrombocytes and finally lymphocytes. However, there is variation within the teleosts on the relative abundance of these cells. As fish do not possess a lymphohaemopoietic bone marrow, blood cell formation occurs in distinct organs that share structural resemblances to the bone marrow of higher vertebrates (Zapata *et al.*, 1996). These organs can be subdivided in primary lymphoid organs (kidney and thymus) and secondary lymphoid organs (spleen and gut-associated lymphoid tissue (GALT) and the mucosal immune sytem (Zapata *et al.*, 1996).

The capacity of the kidney to provide the environment for differentiation of blood cell precursors supports its phylogenetic relationship to the bone marrow of higher vertebrates. It is an important lymphoid organ in teleosts and can be subdivided in two regions, the anterior kidney and the middle and posterior kidney, although both regions exhibit

haemopoietic capacity and are basically structurally similar, with some difference between species (Zapata *et al.*, 1996). There is also evidence for the presence of antigen presenting cells and T-like and B-like lymphocytes in the teleost kidney indicating that the renal lymphoid tissue is an active part of the defence system (Zapata *et al.*, 1996). The spleen is probably the major secondary peripheral lymphoid organ. It is a large blood filtering organ that undergoes increasing structural complexity in order to augment its efficiency in trapping and processing antigens. However, in teleosts with abundant lymphohaemopoietic tissue in the kidney, the splenic lymphoid tissue is poorly developed.

Like mammals, the immune response of teleosts can be subdivided into non-specific and specific immunity. However, the non-specific defence system plays a more important role in fish relative to mammals particularly as the specific defence system may be inactivated at low temperature (Avtalion, 1981). The non-specific cellular mechanisms involve a variety of cells including macrophages, granulocytes non-specific cytotoxic cells and in some cases thrombocytes. These cells are responsible for important defence mechanisms such as inflammation, phagocytosis, pinocytosis, antigen processing and presentation, and non-specific cytotoxicity against cell lines, virus infected cells or protozoan parasites (Secombes, 1996). The humoral non-specific defence mechanism includes lysozyme, complement, interferon, C-reactive protein, transferrin and lectins. Lysozyme is one of the humoral defence factors against invasion by microorganisms. It is an enzyme secreted into the blood by phagocytic cells such as macrophages or neutrophils which is able to split the β(1-4) linkages between the N-acetylmuramic acid and N-acetylglucosamine in the cell walls (peptidoglycan layer) of Gram positive bacteria (Yano, 1996). However purified fish lysozyme is known to be capable of lysing various gram negative pathogens directly without prior attack by complement (Grinde, 1989).

Finally the specific immune system includes specific cell mediated immunity and humoral defence with antibody molecules. The ability of T and B lymphocytes to proliferate will be the only parameter of specific immune response to be studied in the present work and will be induced by Concanavalin A, a T or B cell mitogen, pokeweed, a T cell mitogen, and lipopolysacharide a B cell mitogen.

1.1.2 - Modulation of the immune response

Changes can occur in the immune system of fish as a result of natural factors such as ageing, environmental factors, such as pollutants, or injected stimulants such as vaccines or adjuvants. However, natural routes such as the dietary route can also be utilised to enhance the immune response of fish. In that respect vitamins have been the subject of numerous investigations for their ability to enhance the immune response in fish. Amongst them, vitamin C, a water-soluble, and vitamin E, a liposoluble vitamin, seem to be the most frequently used in fish nutrition and have been shown to enhance some of the immune parameters in various fish species. Both vitamins are known as antioxidants of the cell and seem to be multiple cell stimulators (vitamin C) or B- and T- lymphocyte stimulators (Anderson, 1996).

This stimulation of the immune response is particularly relevant to some aquaculture species where fish reared at higher stocking densities seem to be more susceptible to disease. Section (1.2) will review the role of vitamin E in nutrition and immunomodulation in various animal species. Vitamin E plays a role in prevention of lipoperoxidation and is highly linked and may interact with the lipids with which it is included. The ensuing section (1.3) will therefore evaluate the role of lipids in nutrition, their possible interaction with vitamin E and their role as immunomodulators.

1.1.3 - The turbot (*Scophthalmus maximus*)

Turbot (*Scophthalmus maximus*) is a marine flatfish which belongs to the order Pleuronectiformes and the family Scophthalmidae and is one of the most commercially valuable marine food-fish and one of the most promising sea-fish for intensive culture. When fish are exposed to farming conditions they can suffer from stress which can lead to immunodepression (Anderson, 1990), and studies on the dietary modulation of their immune systems can be particularly valuable.

The turbot is relatively abundant in the North Sea, the Baltic and off the coast of Icelandic (Person-Le Ruyet, 1990). They extend to 68° north along the coast of Scandinavia and are

common off the Moroccan coast to the south; they are also found in the Mediterranean sea (Person-Le Ruyet, 1990). At hatching, turbot larvae are 2.1-2.8 mm in length are planktonic and can be found within 10 meters of the surface. Currents carry them nearer to the shore and at the end of the larval phase the fish undergo metamorphosis, develop asymmetry and descend to the bottom. Then at the juvenile stage the young fish start a benthic existence on intertidal nursery grounds where they remain for 8 months before heading for deeper waters. They will become sexually mature and start their adult life when they are four years old (Person-Le Ruyet, 1990).

They are a carnivorous species which eat small crustaceans at the beginning of their lives but exclusively teleost fish and cephalopods at their adult age (Jones, 1970; Deniel, 1974). The protein requirement of turbot is quite high in quality and quantity whereas small amounts of fat are necessary providing the (n-3) fatty acid requirements are satisfied (Cowey *et al.*, 1976; Gatesoupe *et al.*, 1977). Carbohydrates do not seem to be so important in the diet of turbot due to their problems of utilisation (Jollivet *et al.*, 1988).

Plate 1.1 displays a typical profile of a turbot (*Scophthalmus maximus*)

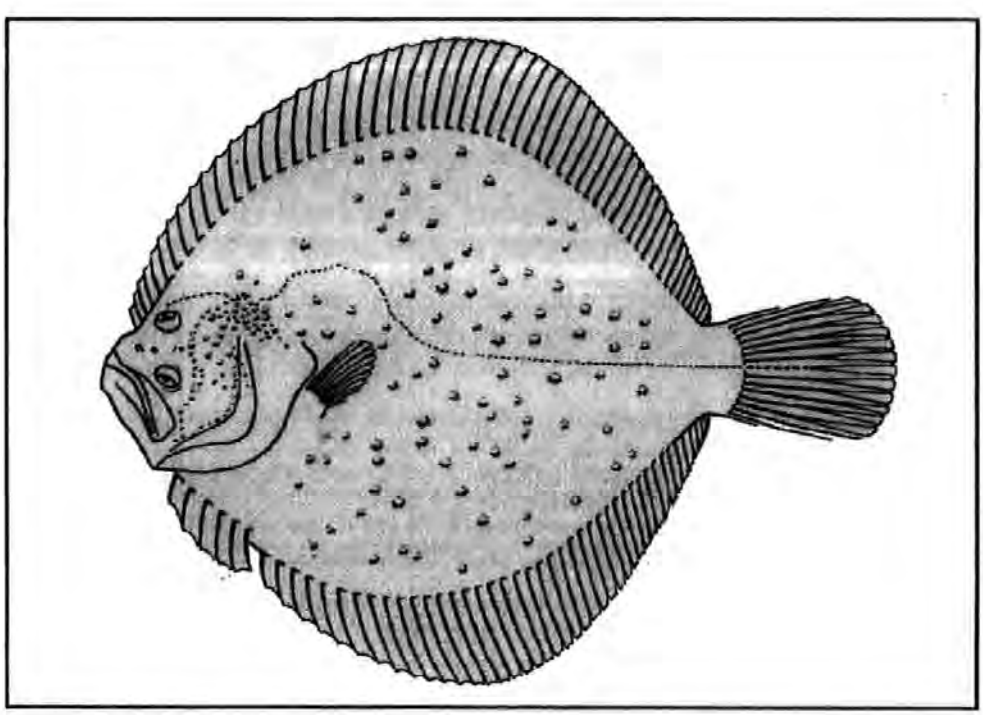


Plate 1.1 : *Scophthalmus maximus* (Linnaeus) (From Norman, 1934)

1.2 - VITAMIN E AND THE IMMUNE SYSTEM

This section will review the discovery, structure, and biological roles of vitamin E and its effect on the immune system of mammals and fish.

1.2.1 - Vitamin E

1.2.1.1 - Discovery and structure

The existence of an antisterility factor was shown by Evans and Bishop in 1922 while rearing rats on a dietary regime consisting of purified proteins, fat and carbohydrate to which an appropriate salt mixture and adequate doses of the growth vitamins, fat soluble A and water soluble B, had been added. The authors observed perfectly normal growth but a sterility problem appeared partly in the first generation and completely in the second generation. They further demonstrated that this problem was not due to a deficiency in vitamin A or vitamin C, but caused by the deficiency of an unknown nutritional factor they named substance X. A series of experiments by Sure (1924) again demonstrated the existence of a specific vitamin for reproduction for which he proposed the name vitamin E instead of substance X. This compound was later to be named tocopherol from the Greek *tocos* meaning childbirth and *pherein* to bring forth, with the *ol* termination indicating the alcoholic nature of the molecule.

Since these early studies, subsequent work has led to the isolation and characterisation of the compound responsible for the restoration of fertility and a more precise definition of deficiency signs in experimental animals. These include reproductive failure, necrotising myopathy, liver and kidney damage and neurological abnormalities (Bender, 1992). Recently the effect of vitamin E deficiency on rat testis and epididymis were characterised (Bensoussan *et al.*, 1998). Vitamin E depletion causes incomlete spermatogenesis and affects the structural differentiation of epithelial cells in the epididymis The deficiency in humans seems to occur only in premature infants of very low birth weight and in patients with abnormalities of lipid absorption or congenital lack of β-lipoproteins (Bender, 1992). In fish, signs of deficiency include erythrocyte fragility closely followed by anaemia,

ascites, xerophthalmia, poor growth, poor food conversion, epicarditis and ceroid deposits in spleen and liver (Halver, 1972).

Vitamin E has eight vitamers, different chemical compounds which show the same biological activity, and which can be further subdivided into tocopherols or tocotrienols depending on whether the side chain is saturated or unsaturated respectively.

Fig. 1.1 represents the 8 vitamers of vitamin E with tocopherol on the left side and tocotrienol with the unsaturated side chain on the right hand side.

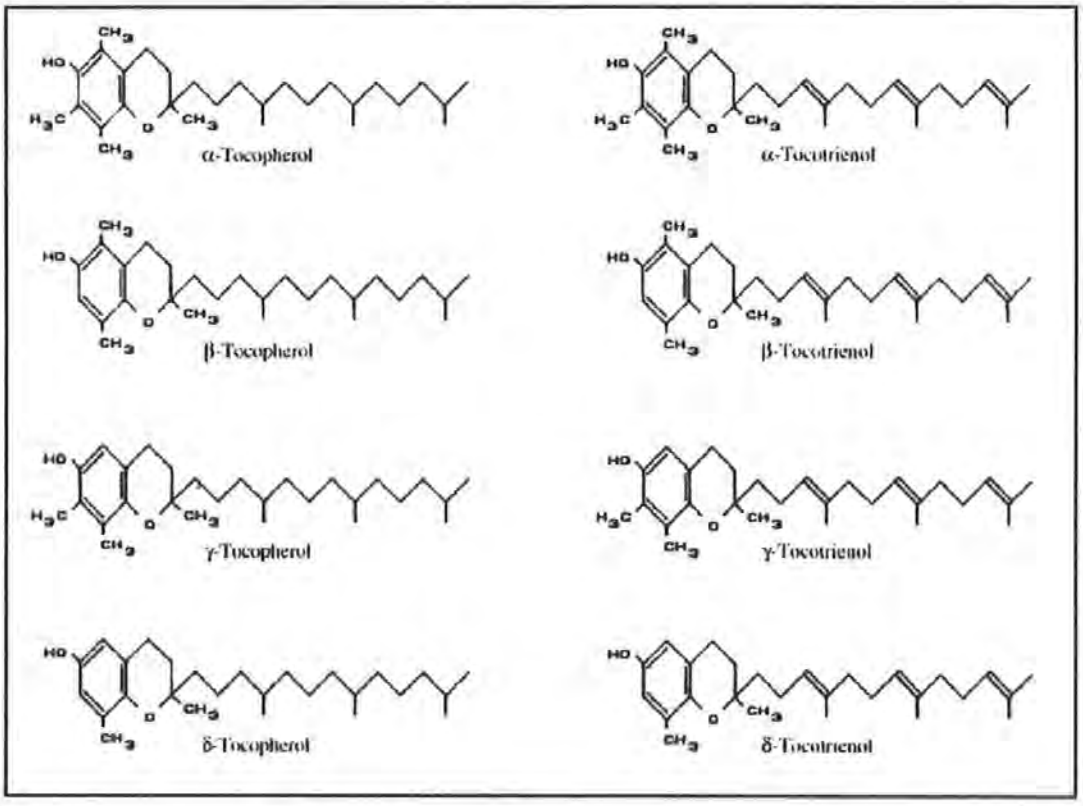


Fig. 1.1: The 8 vitamers of vitamin E (from Bender, 1992)

Tocopherols are composed of a chromanol ring, whose methylation varies giving rise to the different kinds (α, β, γ and δ), associated with a saturated side chain of 13 carbon atoms for tocopherols or an unsaturated side chain for the tocotrienols.

Of all the vitamers α-tocopherol seems to be the most potent and the original international unit of vitamin E potency was even equated with the activity of 1 mg of synthetic DL-α-tocopherol acetate. On this basis pure D-α-tocopherol (*RRR*-α-tocopherol) is the more potent vitamer with 1.49 iu/mg (Bender, 1992). In biological assays, the test substance generally used is DL-α-tocopherol acetate as it is more stable and less susceptible to oxidation than the free D-α-tocopherol (Bender, 1992). The esters are commonly used as

dietary supplements anticipating hydrolysis in the gut and absorption of the free alcohol to act as an active intra- and inter-cellular antioxidant. The structure of α-tocopherol acetate and 2 stereo-isomers of α-tocopherol is represented in Fig. 1.2.

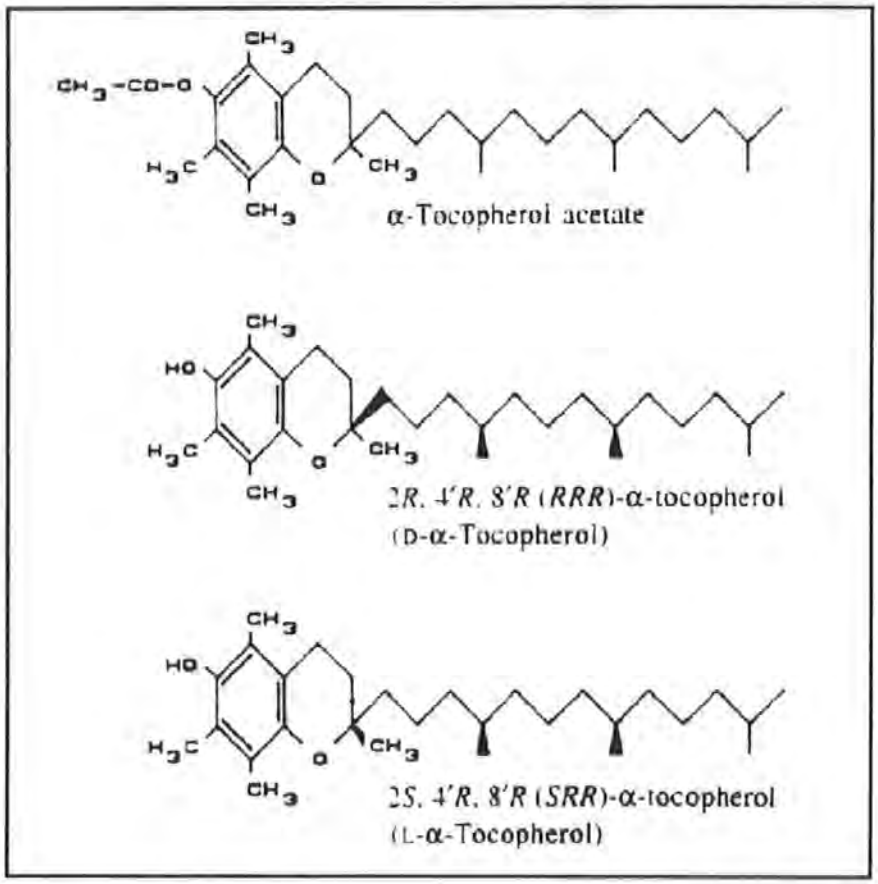


Fig. 1.2 : α-tocopherol acetate and two stereo-isomers of α-tocopherol (from Bender, 1992)

1.2.1.2 - Sources and requirements

Vitamin E is biosynthesised in plants where it is concentrated in chloroplast membranes in green plants and also in large amounts in seeds. Wheat germ, soya beans, eggs, vegetable oils, lettuce and other green vegetables all supply vitamin E.

It is difficult to determine vitamin E requirements in adult humans as when fed on a vitamin E deficient diet they do not develop symptoms of deficiency. Even in young animals it is difficult to totally deplete vitamin E from tissue, even after prolonged vitamin E deficiency. Two explanations advanced by Packer and Landvik (1989) are that either redistribution of vitamin E occurs between tissues and/or oxidised products like tocopheroxyl radical may be regenerated.

However, signs of deficiency shown in various animals, although with considerable differences between species, clearly indicate a necessary vitamin E supplementation in the diet. In humans, average intakes around 8 to 12 mg α-tocopherol equivalent/day seems adequate to meet requirements and maintain adequate plasma concentrations of the vitamin (Bender, 1992). It is however important to note that vitamin E requirements can be influenced by other dietary components especially polyunsaturated fatty acids (PUFAs). Diets rich in highly PUFAs increase the vitamin E requirement due to increased susceptibility of peroxidation of such lipids. It is generally agreed that 0.4 - 0.5 mg vitamin E/g of dietary polyunsaturated fatty acids is adequate but normally food rich in PUFAs are also naturally a good source of tocopherols and tocotrienols (Bender, 1992).

1.2.1.3 - Metabolism of vitamin E

Tocopherol esters are hydrolysed to free tocopherol in the intestinal lumen and mucosa before being absorbed in the intestinal mucosal cells as chylomicrons. These globules are transported to the mesenteric lymphatic and hence into the systemic circulation. Vitamin E is then taken up by the liver which is the main storage organ, although it may also accumulate in the adipose tissue. Thereafter, vitamin E is released into the circulation inside very low density lipoproteins (VLDLs) by the liver. VLDLs are subsequently metabolised in the circulation into low density lipoproteins (LDLs) and high density lipoproteins (HDLs). The vitamin E is then incorporated into erythrocyte membranes where it seems to be in equilibrium with plasma vitamin E (Bender, 1992).

There appear to be two mechanisms by which vitamin E can be taken up by tissues. Either it is released from triglycerides by lipoprotein lipase action or there can be an uptake of LDL-bound vitamin E via LDL receptors (Bender, 1992). Cohn and Kuhn (1989) have studied the role of the LDL receptor for α-tocopherol delivery to tissue in two groups of rabbits, one of which was deficient in LDL receptors. They found an accumulation of α-tocopherol in the plasma of the LDL receptor deficient rabbits suggesting that LDL receptors are the normal delivery route of vitamin E. However, in the absence of LDL receptors none of the tissue investigated was found to be vitamin E deficient probably indicating another route of uptake.

The vitamin E in tissue is mostly found in membranes associated with lipids. Following its absorption and uptake in the tissue, it can be oxidised before being excreted in small amounts in the urine. However, the major route of excretion remains via bile, where it is excreted as unidentified metabolites. Some is excreted through the skin (Bender, 1992).

1.2.2 - Biochemical functions of vitamin E

The main role of vitamin E can be attributed to its ability to protect biological systems against oxidative damage and free radicals. But before examining how it can protect against this damage a brief overview of the mechanisms underlying lipid peroxidation and its relevance to biological systems will be undertaken.

1.2.2.1 - Lipid peroxidation

Lipid peroxidation is a succession of reactions by which polyunsaturated fatty acids (PUFAs) undergo oxidative deterioration. The initiation of this process starts with the attack of PUFAs by a species which has sufficient reactivity to abstract a hydrogen atom from a methylene group of the PUFA being attacked. This attack can be, for example, performed by a free radical defined by Halliwell and Gutteridge (1985) as *any species capable of independent existence that contains one or more unpaired electrons.*

The lipid peroxidation process, once initiated, is followed by different steps represented in Fig. 1.3. The hydrogen abstraction leads to the formation of a carbon radical characterised by an unpaired electron on the carbon from which the H has been abstracted. This induces a rearrangement of the molecule leading to a conjugated diene which then reacts and picks up an oxygen molecule to form a peroxy-radical R-OO$^{\cdot}$. This peroxy-radical can then abstract a H atom from another lipid molecule, leading to the initiation of a chain reaction. The peroxy-radical combines with the hydrogen atom it abstracts from another fatty acid to form a lipid hydroperoxide, or it can also form a cycloperoxide. Lipid hydroperoxides, although fairly stable, can be further transformed into other small compounds in the presence of transition-metal complexes. For example, a lipid peroxide can react with a

reduced iron compound to form an alkoxy radical which is more reactive than the lipid peroxide it came from and therefore can lead to more damage by stimulating the chain reaction again. These reactions can result in the fabrication of different products potentially dangerous for the cell including hydrocarbons or aldehydes such as malonaldehyde.

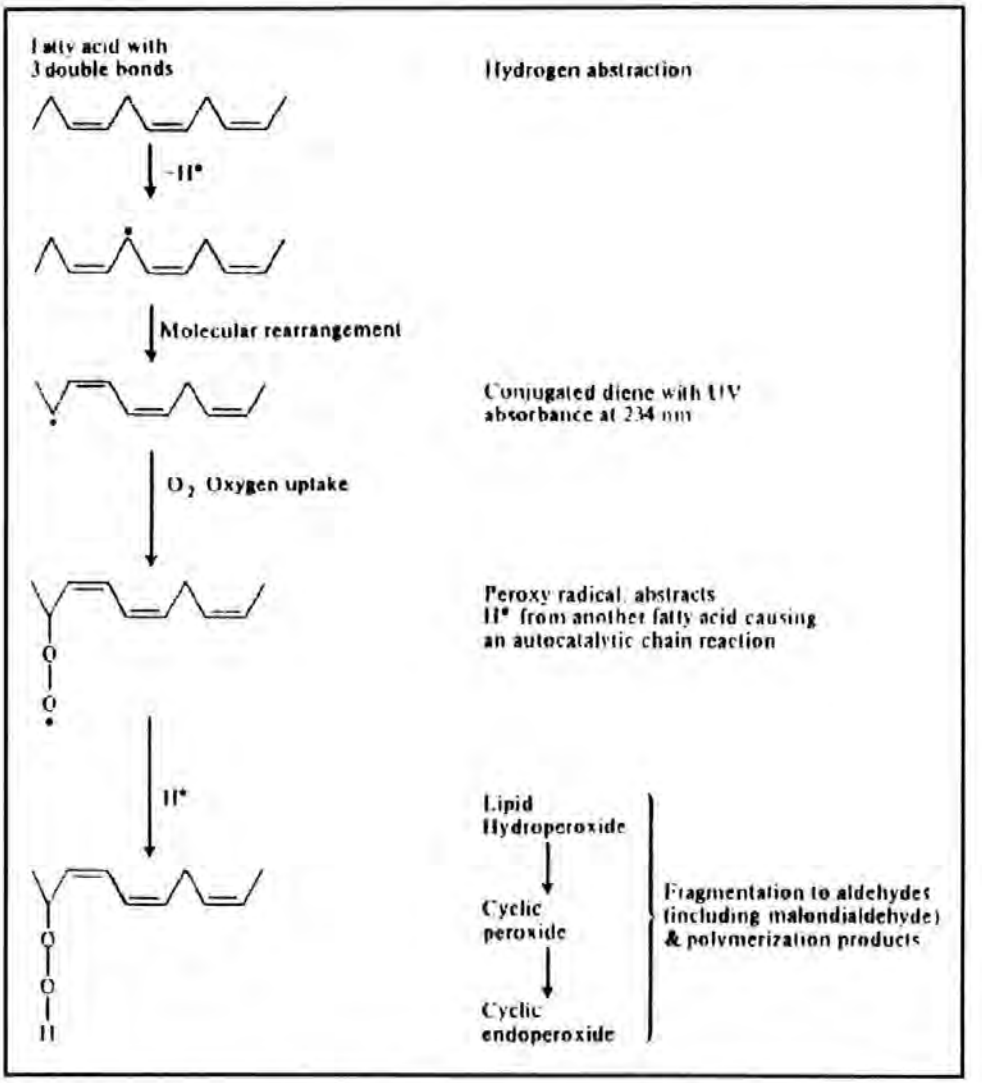


Fig. 1.3 : Initiation and propagation reactions of lipid peroxidation (from Halliwell and Gutteridge,1985)

The problem of lipid peroxidation is of primary importance when one considers the role played by lipids in the cell membrane. The lipid bilayer is the basic structure of a cell membrane and any damage to PUFAs in the membrane will tend to reduce membrane fluidity and therefore affect the correct functioning of biological membranes. This may not be restricted to lipids as the propagation of lipid peroxidation in the membrane may also

affect protein which may be damaged by products of lipid peroxidation. This can lead to enzyme inactivation and further disturbance of physiological processes.

The effect of lipid peroxidation in membranes therefore can be quite dramatic, not only at the cellular level, but by the consequences of this process. It can lead to a decrease in membrane fluidity, inactivation of membrane bound enzymes, and an increase in the leakiness of the membrane that can result in a complete loss of membrane integrity in extreme cases. This can mean that interactions between cells can be affected for example by rending a receptor unavailable at the surface of a cell and therefore interrupting a physiological process.

1.2.2.2 - Antioxidant role of vitamin E

Vitamin E is able to react with lipid peroxy radicals to form vitamin E radicals which are more stable and are not able to initiate other steps in the lipid peroxidation process, thereby stopping the chain reaction. This process presented in Figure 1.4, shows that an unpaired electron can be delocalised in the aromatic ring structure of vitamin E therefore increasing the stability of the molecule. These vitamin E radicals are not reactive enough to abstract another H from the membrane lipids.

Alternatively, vitamin E could quench and react with singlet oxygen before these species start attacking membrane lipids, therefore neutralising possible oxidising agents. The position of the hydroxyl group of α-tocopherol in the lipid/water interface shown by Gomez-Fernandez (1989) when studying phospholipid vesicles, is an argument in favour of this mechanism of prevention. Indeed any oxidizing agent approaching the membrane surface will find reducing protons before they can penetrate the membrane. Another investigation on the mode of action of vitamin E on liposomal membranes studied by spin labelling came to similar conclusions that vitamin E scavenges radicals at or near the surface of the membranes more easily than those that reside deep in the bilayer (Takahashi *et al.*, 1989).

It has also been suggested that vitamin E could protect against peroxidation by modifying membrane structure (Halliwell and Gutteridge, 1985). An experiment carried out on phospholipid liposomes prepared either using vitamin E or vitamin E acetate showed that

there was a certain degree of protection against peroxidation when incorporating vitamin E acetate at high levels into the preparation. Vitamin E acetate is the esterified form of the vitamin E molecule which has lost its ability to react with lipid hydroxy radicals, and therefore the protective effect induced was not due to the quenching of radicals but might be attributable to a structural effect induced by its incorporation into phospholipid liposomes (Halliwell and Gutteridge, 1985).

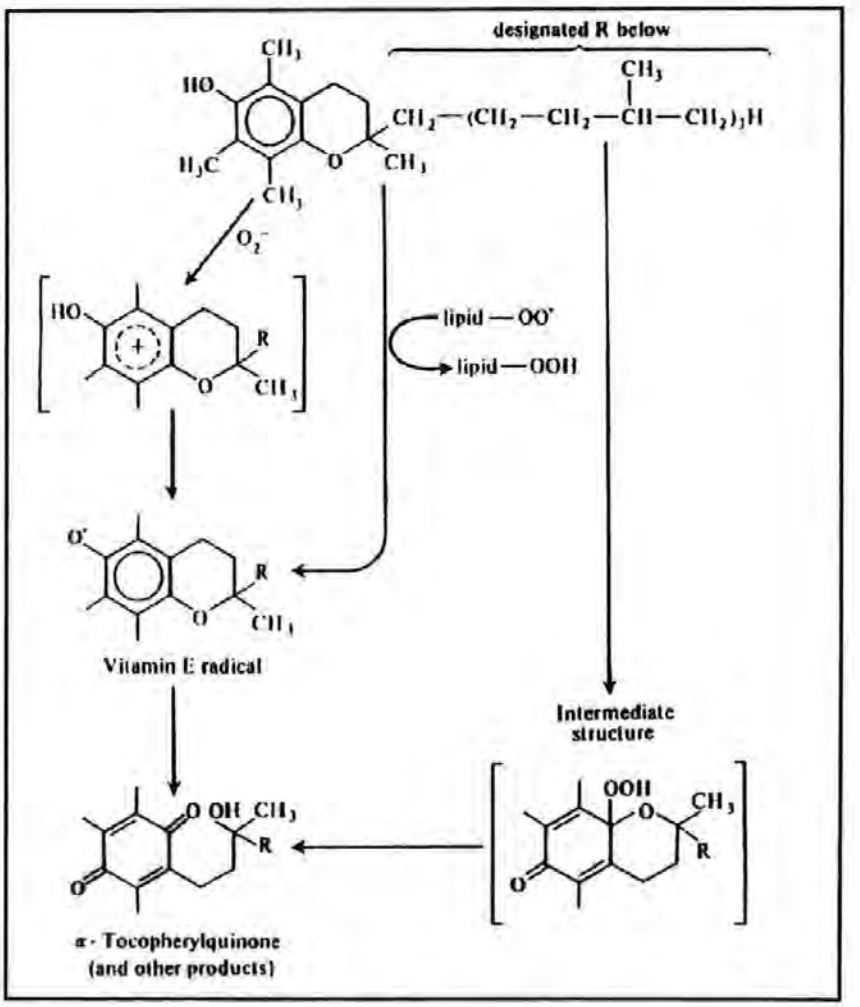


Fig. 1.4 : Prevention of oxidation by vitamin E (from Halliwell and Gutteridge, 1985)

1.2.2.3 - Other functions of vitamin E on membranes

It has been proposed that in addition to its antioxidant role vitamin E might have a specific physico-chemical role in ordering membrane lipids (Bender, 1992). However, it remains difficult to draw a clear separation between these two roles as ordering membrane lipids may result in a better protection of these lipids against oxidative attack, ultimately resulting in protection against lipid peroxidation. It has been advanced that the phytil side

chain of *RRR*-α-tocopherol, by interacting with the methylene-interrupted cis-double bonds of arachidonate and other long chain PUFAs, could stabilise membrane structure. Therefore, α-tocopherol would have an effect in preserving membrane impermeability. Kagan (1989) studied the effect of tocopherol in stabilising membranes against phospholipase A, free fatty acids and lysophospholipids. He concluded that α-tocopherol could prevent or even abolish the disordering effects of free fatty acids on phospholipid bilayers due to the formation of complexes within the membrane core. Thus vitamin E seems to form stable complexes which ensure protection of the membrane against the damaging effect of fatty acids and lysophospholipids.

Fig. 1.5 represents the different biochemical functions attributed to vitamin E. Its antioxidant role is emphasized with its chain-breaking antioxidant and prevention of lipid peroxidation (top left) and its role as a singlet oxygen species quencher (top right). Vitamin E can also modulate the metabolism of arachidonic acid cascade initiated by cyclooxygenase (COX) or lipoxygenase (LO) (bottom left) or order phospholipids in the membrane thus acting on the membrane fluidity (bottom right) and modulating other important enzymes such as protein kinase C.

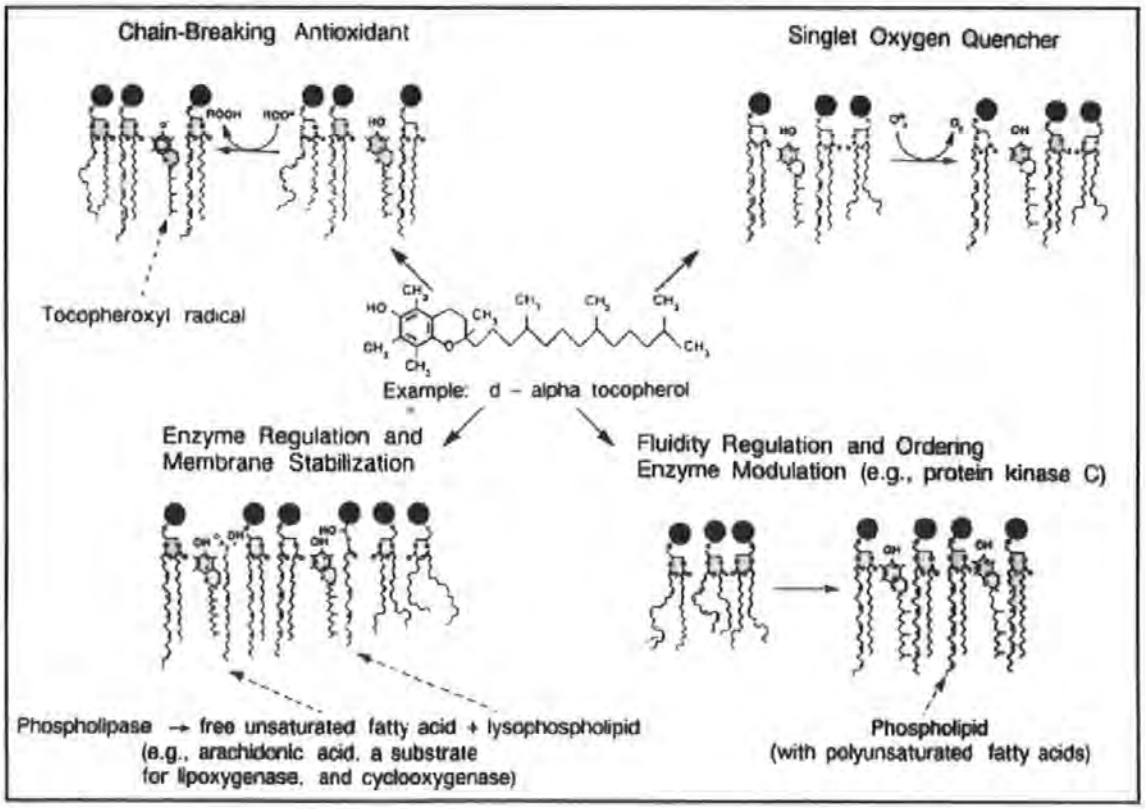


Fig. 1.5 : Different biochemical functions of vitamin E (from Packer and Landvik, 1989)

1.2.2.4 - Interaction of vitamin E and other compounds in the protection against lipid peroxidation

1.2.2.4.1 - Vitamin C

The tocopheroxyl radical formed by the reaction of α-tocopherol with a lipid peroxide can be reduced back to α-tocopherol with the help of another agent. The most famous interaction in that respect seems to be the interaction of vitamin E and vitamin C shown in Fig. 1.6 (Bender, 1992).

Ascorbic acid can indeed reduce the tocopheroxyl radical back to α-tocopherol and itself be transformed into monodehydroascorbate.

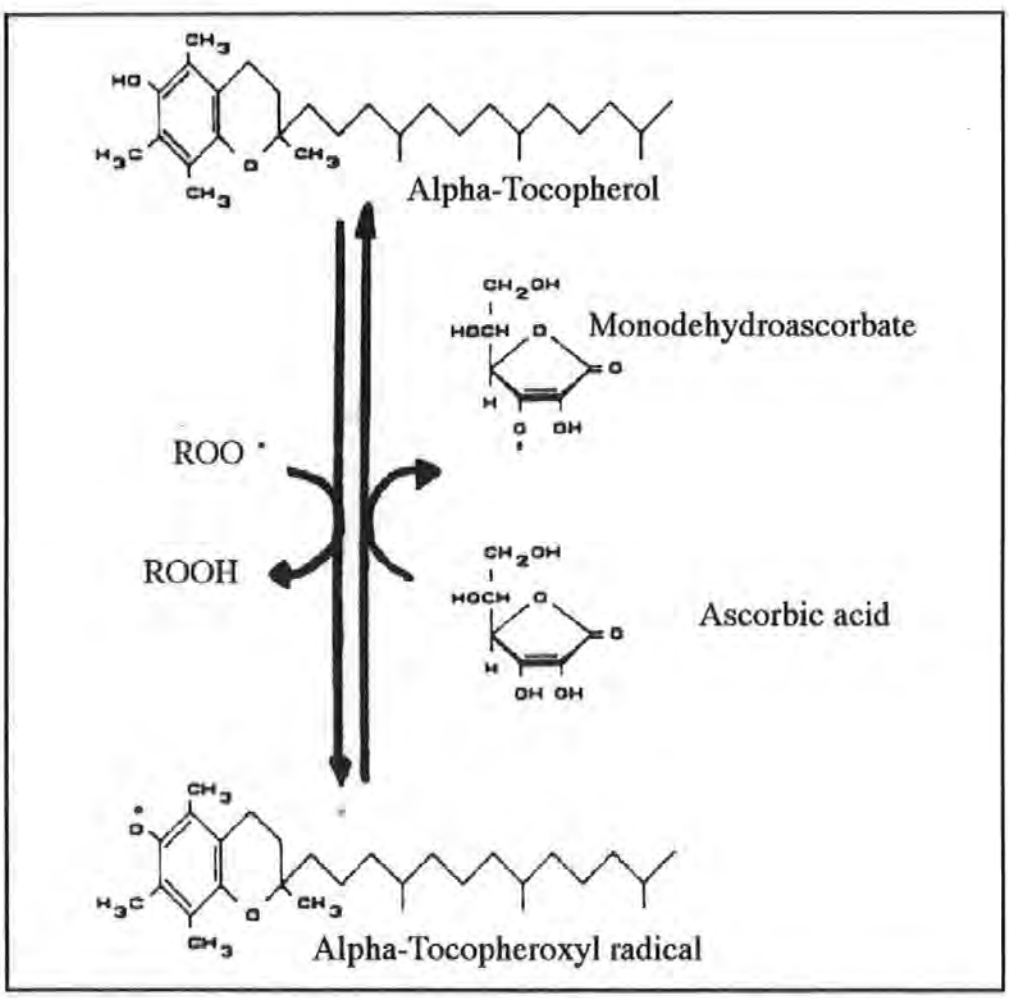


Fig. 1.6. Reduction of tocopheroxyl radical to α-tocopherol by vitamin C (from Bender, 1992)

There appears to be a synergism in the interaction of these two vitamins localised in two different compartments. Vitamin E, a liposoluble vitamin, is localised in the membrane whereas vitamin C, a water soluble vitamin, is localised in the cytosol. Synergistic

inhibition of oxidation has been studied in red blood cell ghosts by Miki *et al.* (1989). They used azo-compounds as oxidising agents and observed that vitamin E in membranes was scavenging the lipid peroxy radical induced by 2, 2' azobis (2, 4-dimethyl-valeronitrile) (AVMN) whereas vitamin C in the aqueous phase was regenerating vitamin E radicals to vitamin E. A sparing action of vitamin C on vitamin E has been demonstrated in studies when different groups of animals showed higher levels of vitamin E when fed higher levels of vitamin C (Yu, 1994). Liu and Lee (1998) also showed that vitamin C supplementation could restore the impaired vitamin E status of guinea pigs fed oxidised frying oil. Finally, May *et al.* (1998) showed that ascorbate within the erythrocytes protects α-tocopherol by a direct recycling mechanism in humans.

1.2.2.4.2 - Selenium

Other compounds seem to facilitate the regeneration of α-tocopherol from α-tocopheroxyl radicals in different systems. Glutathione (GSH) can achieve this reduction with the intervention of a seleno-enzyme: hydroperoxide glutathione peroxidase. Glutathione peroxidase is a selenium containing enzyme found in the cytosol and mitochondria of animal tissues which catalyses the following reaction.

$$2GSH + H_2O_2 \rightarrow 2\ H_2O + GSSG$$

where *GSH* is glutathione and *GSSG* is the oxidised glutathione formed by two molecules of GSH joined together as the -SH groups of cysteine are oxidised to form a disulphide bridge. Glutathione peroxidase is specific for GSH as a substrate, but can transform a range of peroxide (ROOH) *in vitro* other than hydrogen peroxides to reduced alcohols (ROH). Selenium deficiency in animals produces a variety of diseases that are similar to those induced by vitamin E deficiency, and supplementation of vitamin E in selenium deficient animals or vice versa may be beneficial. Thus selenium required at the catalytic site of this enzyme can help not only to regenerate α-tocopherol from tocopheroxyl radical but can also dispose of hydrogen peroxide and so reduce the amount of peroxides available for the generation of radicals.

1.2.2.4.3 - Other mechanisms of regeneration of α-tocopherol in the cell

It has also been shown in mitochondrial membranes that the tocopheroxyl radical can be reduced by an electron transport chain linked enzymic system (Maguire *et al.*, 1989).

1.2.2.5 - Vitamin E and eicosanoid synthesis

In accordance with the potential antioxidant action of vitamin E and its role in prevention of oxidative processes one can expect such compounds to affect the biosynthesis of thromboxane (TX), prostaglandins (PG) and leukotrienes (LT). These compounds are indeed oxygenated derivatives of C_{20} PUFAs via lipoxygenase or cyclooxygenase enzymatic pathways. A few studies have investigated this interaction. Goetzl (1980) showed that vitamin E had a bimodal action upon leucocyte lipoxygenation products of arachidonic acid when added *in vitro*. When human neutrophils were preincubated with 0.03 to 0.06 mM of vitamin E for 20 minutes, the levels of endogenous hydroxyeicosatetraenoic acid (HETE) were consequently enhanced by a mean of two- to three-fold. However, preincubation of such cells with 0.5-1 mM of vitamin E clearly reduced the levels of endogenous HETEs. Goetzl showed that this mechanism was not a function of changes in the rate of mobilisation of arachidonic acid from phospholipids. He also pointed out that vitamin E was modulating human neutrophil migration *in vitro* at concentrations that affect the lipoxygenase pathway but adding HETEs to α-tocopherol treated neutrophils failed to reverse the migration defects. This enabled him to conclude that the effects of α-tocopherol on human neutrophil function were not solely attributable to alterations in the cellular content of endogenous HETEs and may also reflect changes in concentrations of unstable hydroperoxy-eicosatetraenoic acids (OOHETEs) or non-lipoxygenase related actions of vitamin E.

In general a suppression of lipoxygenation of arachidonic acid by vitamin E may lead to immunoenhancement (Tengerdy, 1989). Tengerdy (1989) in a study with chicken showed a similar action of vitamin E and aspirin, a cyclooxygenase inhibitor, on PG production in immunopoietic organs and a decreased mortality when the chickens were infected with *E. coli* after treatment. The protection against disease was total when the two factors, vitamin E and aspirin, were given simultaneously. Meydani (1989) also observed an impact of

vitamin E on eicosanoid synthesis. Aged mice fed with a supplement of 500 ppm of vitamin E for 6 weeks showed increased splenocyte proliferation in response to Con A and LPS that the author correlated with a decrease in PGE_2 synthesis and an increase in IL-2 production. In a study on aged humans, Meydani (1989) observed similar effects of vitamin E supplementation, that is to say an enhanced mitogenic response of lymphocytes to Con A but decreased PGE_2 production by peripheral blood mononuclear cells in the vitamin E treated group. However Sakamoto *et al.* (1996) showed that vitamin E failed to inhibit PGE_2 production in LPS-stimulated rat peritoneal macrophages. This suggests that vitamin E does not inhibit COX expression via LPS- mediated tyrosine kinase signal transduction pathway but it does not exclude an influence of vitamin E on another pathway. In contrast, Wu *et al.* (1998) showed that vitamin E supplementation reversed the increased PGE production in old mice but had no effect on young mice and that it exerted its effects posttranslationally by inhibiting COX activity.

As shown by Tengerdy (1989) other dietary components may interact with vitamin E in the modulation of eicosanoid synthesis. Thus lipids modify the requirements of vitamin E in the diets and can therefore act upon the effective concentration of vitamin E avaiable for modulation of PG production.

1.2.3 - Vitamin E and immune function

There have been extensive studies on vitamin E dietary supplementation in animals and its impact on immune response or disease resistance. This section will be subdivided in two parts, one concerning higher vertebrates mainly mammals and the other focused on marine and freshwater animals. For increased clarity a synthesis of the results will be presented as tables.

1.2.3.1 - Vitamin E and immune competence in higher vertebrates.

The effect of vitamin E supplementation on different animals are listed in table 1.1. Most of these supplementation experiments were carried out by dietary means although some were performed by injection but the routes are specified in the tables.

On the whole vitamin E supplementation in higher vertebrates seems to be beneficial to lymphocyte proliferation stimulated with mitogens, antibody production, disease resistance after challenge with a pathogen and phagocytosis. However, it is important to note that a few studies suggest that although vitamin E supplementation may be beneficial to the number of particles being phagocytosed by macrophages or other phagocytic cells, the successive killing of bacteria may be decreased in highly vitamin E supplemented animals (Panush and Delafuente, 1985). This phenomenon is emphasised in the review of Panush and Delafuente (1985) and comes from two different studies in humans. The reduction in neutrophil bactericidal activity in vitamin E supplemented patients was thought to be caused by a lack of availability of intracellular hydrogen peroxide due to a decrease in the rate of superoxide anion generation (Baehner *et al.*, 1977). In the other study reviewed in this paper the release of acid phosphatase by phagocytosing cells was significantly decreased (Prasad, 1980), while another paper highlights a decreased oxygen consumption by neutrophils phagocytosing bacteria (Repine *et al.*, 1978). So although beneficial on some counts high vitamin E supplementation might be detrimental to some killing mechanisms used by phagocytic cells to dispose of pathogens, especially the ones involving superoxide anion production.

Animal	Dosage	Findings	Reference
Humans	300 mg/day for 3 weeks in the diet	Decreased peripheral blood lymphocyte proliferation in response to PHA	Panush and Delafuente, 1985
Humans	400 IU 4 times daily for 7 days in the diet	Increased neutrophil phagocytic rate Bactericidal capacity decreased	Panush and Delafuente, 1985
Humans	300 mg daily for 3 weeks in the diet	Decrease in bactericidal activity	Panush and Delafuente, 1985
Humans	1200 IU/day	Decrease superoxide anion release from monocytes Decrease release of IL-1 and TNFα from monocytes	Devaraj and Jialal, 1998
Aged humans	400 IU in soybean oil ingested twice daily	Decreased PGE_2 production by PBMC DTH antigen score and cumulative score increased Increased lymphoproliferation to Con A	Meydani *et al.* 1989
Aged humans	60 mg/day	41 % increase in DTH, 3-fold increase in AB titer to hepatitis	Meydani *et al.*, 1997
	200 mg/day	65 % increase in DTH, 6-fold increase in AB titer to hepatitis	
	800 mg/day for 235 days	49 % increase in DTH, 2.5-fold increase in AB titer to hepatitis	
Rats	50mg/kg of diet 200 mg/kg of diet	Increased response of splenocytes to ConA and LPS	Bendich, 1988
Rats	Vitamin E deficiency	Decreased ConA- stimulated lymphocyte proliferation	Pighetti *et al.*, 1998
Mouse	120 mg/kg of diet 300 mg/kg of diet	Increased phagocytosis of diplococci Increased bacterial lysis Increased phagocytosis of carbon particles in the blood	Panush and Delafuente, 1985
Mouse	50 mg/kg of diet 500 mg/kg of diet	Increased spleen cell response to ConA	Panush and Delafuente, 1985

Table 1.1. Effect of vitamin E supplementation on immune competence of higher vertebrates

Animal	Dosage	Findings	Reference
Mouse	200 mg/kg of diet	Increased antibody response to RBC	Panush and Delafuente, 1985
Mouse	180mg/kg 360mg/kg	Increased resistance to *Diplococcus pneumoniae*	Panush and Delafuente, 1985
Mouse	2.035 IU/kg of diet	Increased PFC and HA titers	Nockels, 1979
Mouse	150 IU/litre (15-fold increase)	Increased T-cell proliferation and nhancement of B cell proliferation suppressed by retrovirus at 4, 8, and 12 weeks post-infection with retrovirus IL-2 production restored	Wang *et al.*, 1994
Mouse (young and old)	*In vitro* addition of 4 µg/ml of serum	Enhanced mitogenic response of splenocytes to PHA or ConA	Meydani *et al.* 1989
Mouse (aged)	500 ppm dietary supplementation for 6 weeks	Increased lymphoproliferation with ConA and LPS Increased production of IL-2 and decreased production of PGE_2	Meydani *et al.* 1989
Mouse	200 mg/kg of diet	Increased anti HRBC haemagglutinin titers	Tanaka *et al.* 1979
Guinea pigs	Two 250 IU/kg of body weight injections or 33 IU/kg body weight	Increased antibody response to virus	Nockels, 1979
Sheep	300 IU/kg of diet	Decreased percentage of infection to *Chlamydia*	Nockels, 1979
Chick	150 IU/kg of diet 300 IU/kg of diet	Decreased mortality against *E.coli* HA titers increased	Nockels, 1979
Chickens	0.1 mg/g of body weight injection at two days interval	Enhanced mitogen mediated T lymphocyte proliferation	Kline *et al.* 1989
Chickens	300 mg/kg of diet	Decreased mortality against *E.coli*	Tengerdy, 1990

Table 1.1 (continued). Effect of vitamin E supplementation on immune competence of higher vertebrates.

Animal	Dosage	Findings	Reference
Chicks	60 mg/pound of diet	Enhancement of antibody production	Panush and Delafuente, 1985
Turkey	100 IU/kg of diet 300 IU/kg of diet	HA titers increased for 300 IU Decreased mortality against *E.coli*	Nockels, 1979

Table 1.1 (continued). Effect of vitamin E supplementation on immune competence of higher vertebrates.

1.2.3.2 - Vitamin E and immune competence in fish

The main findings of dietary vitamin E influence in fish are listed in the table 1.2.

A noticeable difference between higher vertebrates and fish in the influence of vitamin E supplementation on immune competence seems to be the prevalence of the deleterious effect of vitamin E depletion in fish compared to the beneficial effects of vitamin E supplementation in higher vertebrates. Except for a few studies (N'Doye, 1993; Verlhac and Gabaudan, 1997) where vitamin E supplementation has a positive effect on rainbow trout (*Oncorhynchus mykiss*) immune response, it is noteworthy that vitamin E depletion is the main cause of dysfunction or negative modulation of the immune response in fish. Most of the studies carried out with various vitamin E doses failed to reveal any stimulatory effect of high doses of vitamin E compared to control treatments, whereas they did in most of the studies using higher vertebrates as experimental animals. However, the experiments with higher vertebrates also showed negative immunomodulation when fed vitamin E deficient diets.

Furthermore, a feature common to higher vertebrates and fish is the negative influence of high vitamin E supplementation on killing mechanisms of phagocytic cells as noted in the study by Blazer (1991). This author is in agreement with other reviewers on higher vertebrates and explains this phenomenon by the fact that excessive doses may reduce the intracellular killing ability, if that killing depends on peroxidative damage to engulfed organisms.

Animal	Dosage	Findings	Reference
Carp *Cyprinus carpio*	Deficiency : 0 mg/kg for 17 weeks	Decreased white blood cell counts	Watanabe *et al.* 1970a
Rainbow trout *Oncorhynchus mykiss*	Depleted diets for 12-17 weeks	Depressed immune response to SRBC and to *Yersinia ruckeri* Decreased phagocytic index of peritoneal macrophages	Blazer and Wolke 1984
Rainbow trout *Oncorhynchus mykiss* (W)	806 mg/kg of diet	Decreased mortality following bath challenge or intra peritoneal injection with *Yersinia ruckeri*	Furones *et al.* 1992
Rainbow trout *Oncorhynchus mykiss* (W)	Deficiency 0 mg/kg of diet	Decreased haematocrit and increased haemolysis	Frischknecht *et al.* 1994
Rainbow trout *Oncorhynchus mykiss*	600 mg/kg of diet for 16 weeks	Antibody production after vaccination against enteric red mouth disease increased Increased peripheral blood lymphocyte proliferation with LPS Increased phagocytic index of yeast cells Increased pinocytosis of neutral red Increased oxidative burst with lucigenin used as a substrate	Verlhac and Gabaudan, 1997
Rainbow trout *Oncorhynchus mykiss*	450 mg/kg of diet	Antibody production after vaccination against *Yersinia ruckeri* stimulated Stimulation of peripheral blood lymphocyte proliferation with PHA, ConA, and PWM Stimulation of phagocytosis	N'Doye, 1993
Chinook salmon *Oncorhynchus tshawytscha*	Low dietary level 5.7 mg/kg of diet	Decreased haematocrit value	Thorarinsson *et al.* 1994
Atlantic salmon *Salmo salar* L.	Deficiency	Increased mortality following *Aeromonas salmonicida* challenge Complement activity compromised	Hardie *et al.* 1990
Atlantic salmon *Salmo salar* L.	Vitamin E supplementation	No effect on non-specific immune resistance to *Aeromonas salmonicida*	Lall *et al.* 1988

Table 1.2. Effect of vitamin E supplementation or deficiency on immune competence of different fish species

Animal	Dosage	Findings	Reference
Channel catfish *Ictalurus punctatus*	Deficiency 0 mg/kg of diet	Decreased ability for macrophages to phagocytose *Edwarsiela ictaluri*	Wise *et al.* 1993b
Channel catfish *Ictalurus punctatus*	Deficiency 0 mg/kg of diet 2,500mg/kg of diet	Lower phagocytic index Reduced killing index Killing index decreased	Blazer, 1991

Table 1.2 (continued). Effect of vitamin E supplementation or deficiency on immune competence of different fish species

1.2.3.3 - Interaction of vitamin E with other nutrients and its effect on immune competence.

From the observation that dietary vitamin E requirements vary according to the composition of the diets, particularly regarding fat composition, a certain number of studies have focused on the interaction of vitamin E and polyunsaturated fatty acids in the diet. Some of these studies are reviewed in the paragraph on lipids and immune response (1.346) and another group of studies focussed on the interaction of dietary oxidised or non-oxidised oil and vitamin E on the immune system will be presented here. As vitamin E interacts with other elements or vitamins in its role of protection against peroxidation (vitamin C or selenium) a few studies were carried out on such interactions in order to determine which dietary intake would best optimize an immune response ; these are briefly reviewed in this section.

1.2.3.3.1 - Vitamin E and oxidised oil

Juvenile coho salmon (*Oncorhynchus kisutch*) were fed on diets containing oxidised herring oil and low or high levels of vitamin E (Forster *et al.*, 1988). The fish were either vaccinated or not against *Vibrio* and were subsequently submitted to challenge with *Vibrio anguillarum* or *V. ordali*. In either case, diet did not affect the response of coho salmon to pathogens. Rainbow trout (*Oncorhynchus mykiss*) studied by Moccia *et al.*, (1984)

exhibited lower haematocrit and increased haemolysis when fed highly or extremely oxidised oil and depleted vitamin E levels. Two other studies by Obach and Baudin Laurencin (1992) and Obach *et al.* (1993) focused on immunomodulation by dietary oil and vitamin E. The first revealed that turbot (*Scophthalmus maximus*) fed for 9 months with diets containing oxidised or fresh oil and depleted or low suplementation of vitamin E were affected in some of the parameters measured. The mean mortality rate after challenge with *Vibrio anguillarum* was increased in fish fed with the oxidised oil and the vitamin E free diet but the agglutinin titers were not affected by the diet. This led the author to conclude that the origin of the changes could be in the alteration of humoral non-specific or cellular mechanisms of defence. The respiratory burst activity measured by chemiluminescence was also affected by the oxidised and vitamin E free diet. This was correlated with high levels of TBA-reacting substances in the muscle, supposed to reflect the degree of lipid peroxidation *in vivo*, and the hypothesis of impairment of phagocytosis by these peroxidative processes was advanced by the author. The second study by the same author (Obach, 1993) was carried out on the interaction of dietary α-tocopherol and oxidised oil in sea-bass (*Dicentrarchus labrax*). Fish were fed for 35 weeks on 6 different regimes varying with the inclusion of different levels of vitamin E (0, 40 or 300 mg/kg of diet) and the quality of the oil, fresh or oxidized. Fish were subsequently tested for different blood parameters and both specific and non-specific immune parameters were measured. Unlike the experiment with turbot (Obach and Baudin-Laurencin, 1992) challenge with *Vibrio anguillarum* led to high levels of mortalities (78% to 90%) but no differences between the dietary treatments could be demonstrated. The antibody response among fish did not vary according to the diets but chemiluminescence of head kidney phagocytes stimulated by opsonised zymosan was lower for fish fed oxidised oil and vitamin E free diets compared to those fed oxidised oil and 300 mg of vitamin E, or fresh oil and 40 mg of vitamin E. In this experiment complement activity and serum lysozyme were measured and these factors were both affected by the oil quality and vitamin E. Lysozyme activity was higher for groups fed fresh oil and vitamin E supplemented diets and complement activity was higher for fish fed fresh oil and high vitamin E. However, this change in complement activity did not influence the resistance to disease of such fish as no differences in mortalities were observed between dietary treatments.

1.2.3.3.2 - Vitamin E, vitamin C and selenium

Bendich (1988) reviewed the interaction of vitamin E and C on immune response of experimental animals. Guinea pigs, like man, are unable to synthesize vitamin C *de novo* and were chosen as the experimental subject. Animals were fed on a group of diets with 0, 30 or 200 mg vitamin E/kg and low or high levels of vitamin C. When Guinea pigs were exposed to 100% oxygen the animals fed vitamin E deficient diets and high vitamin C showed enhanced T and B mitogen responses compared to the group fed the low vitamin C diet. The animals fed with high levels of both vitamins showed the best response amongst the different groups. Frischknecht *et al.* (1994) studied the pathological changes due to vitamin E and C in rainbow trout (*Oncorhynchus mykiss* [Walbaum]). The fish fed a diet deficient in both vitamins were anaemic and exhibited high mortalities whereas fish given supplements of both vitamin E and C did not present any gross necroscopy signs as did fish fed with a diet deficient in one vitamin or the other.

Bendich (1988) also reported various experiments where selenium and vitamin E were studied in parallel for their effect on immune competence of different laboratory animals. The results obtained varied according to the species but overall selenium is not convincing in its ability to substitute for vitamin E or even act synergistically with vitamin E. A study on rats reported that animals fed for 4-8 weeks with a vitamin E and selenium deficient diet had severely depressed T- and B- lymphocyte responses to mitogens. The supplementation of such diets with selenium did not enhance the response whereas supplementing the diet with 200 mg of vitamin E/kg of diet did so. When both vitamin E and selenium were added to the diet no further enhancement was found. Another study on rats reported in this review (Eskew *et al.*, 1985) points out an increase of mitogen response in rats when diets were supplemented with both selenium and vitamin E greater than the increase seen when either nutrient alone was added to the diet.

Channel catfish have been studied by Wise *et al.* (1993a) for the influence of dietary selenium and vitamin E upon macrophage superoxide anion production. The extracellular secretion of superoxide anion by PMA-stimulated macrophages was not affected by the experimental diets. On the other hand macrophages taken from fish fed on high vitamin E and selenium levels stimulated by phagocytosis with *E.coli* demonstrated significantly higher production of intracellular superoxide anion. This phenomenon, although rather

surprising, was thought to reflect the enhanced phagocytic ability. When considering all parameters measured in the experiment the authors concluded that the nutrients were not complementing each other or that one was not compensating for the deficiency of the other. This dietary interaction was also studied by Thorarinsson *et al.* (1994) in chinook salmon (*Oncorhynchus tshawytscha*) for susceptibility to infection. Although no definite effect of dietary selenium and vitamin E on the prevalance and severity of natural *Renibacterium salmoninarum* infections was demonstrated, no mortality was noted in the group of fish fed with high levels of both nutrients as opposed to 31% mortality in fish fed the diet deficient in both nutrients and 3% in fish fed the diets supplemented with either nutrient.

1.3 - LIPIDS AND THE IMMUNE SYSTEM

This section is compiled from the following books Beck *et al.* (1991), Stryer (1995), Becker and Deamer (1991). Additional material has been incorporated and will be acknowledged where appropriate.

1.3.1 - Lipids

1.3.1.1 - Nomenclature

Lipids are a heterogeneous collection (fats, oils, waxes and related compounds) of organic substances that are insoluble or sparingly soluble in water. They can be subdivided into triglycerides which are simple lipids or neutral lipids and serve as an energy store in the cells, phospholipids which are important components of the cell membrane, and steroids a peculiar category of lipids including diverse molecules such as cholesterol, vitamin D and a variety of hormones.

Triglycerides are important constituents of the body and are synthesised by a condensation reaction of one molecule of glycerol and three molecules of fatty acid ($CH_3(CH_2)_nCOOH$, where (n) is generally an even number). Fatty acids have two functional groups, a carboxyl group (-COOH) at one end which is polar and therefore water-soluble, and a long chain

composed solely of hydrogen and carbon atoms which is therefore non-polar and hydrophobic. This double-sided character accounts for certain characteristics of the lipids and for their interface role in immiscible mixtures of water and oil, for example in the lipid layer in biological membranes.

Fatty acids are named after the parent hydrocarbon by the substitution of *oic* for the final *e*. For example the C_{18} saturated fatty acid is called octadecanoic acid because its parent hydrocarbon is octadecane. A C_{18} fatty acid with one double bond will be named octadec*enoic* acid from the corresponding hydrocarbon with one double bond octadec*ene*. Carbon atoms are numbered from the carboxyl end of the molecule with carbon atoms 2 and 3 referred to as α and β respectively. The methyl carbon atom at the distal end of the chain is called the ω carbon or also noted n. Fig. 1.7 represents the structure of a fatty acid molecule.

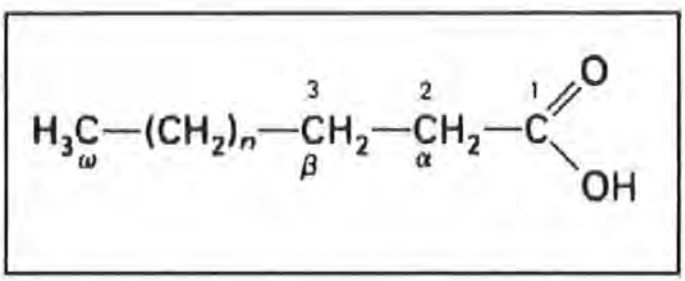


Fig. 1.7: An example of fatty acid molecule (from Stryer, 1995)

1.3.1.2 - Classes of fatty acids

Fatty acids are divided in two classes whether or not the carbon chain carries the maximum possible number of attached hydrogens: the saturated fatty acids which have the maximum number of hydrogens, and the unsaturated fatty acids which have double bonds joining the carbon atoms that are not fully saturated with hydrogen.

Saturated fatty acids originate in animal fats and can be synthesised in mammalian tissue, these types of fat are solid at room temperature. These saturated fatty acids can have an infinite number of arrangements for their three dimensional structure because each carbon-carbon bond has complete freedom of rotation. However, the second group of fatty acids, the unsaturated fatty acids, has a rigid kink in their chains because double bonds between carbons cannot rotate. Their carbon chains have a fixed kink at each double bond which

makes them behave differently from saturated fatty acids in membranes, disrupting the close packing that is possible with saturated fatty acids. Such fatty acids are found in fats which are liquid at room temperature and are termed oils. Fatty acids containing more than one double bond in their carbon chain are called polyunsaturated fatty acids (PUFAs). The position of a double bond is represented by the symbol Δ followed by a superscript number indicating the carbon atom number at which the double bond is located. For instance Δ^9 means there is a double bond between carbon atoms 9 and 10.

Number of carbons	Number of double bonds	Common name	Systematic name	Formula
12	0	Laurate	n-Dodecanoate	$CH_3(CH_2)_{10}COO$-
14	0	Myristate	n-Tetradecanoate	$CH_3(CH_2)_{12}COO$-
16	0	Palmitate	n-Hexadecanoate	$CH_3(CH_2)_{14}COO$-
18	0	Stearate	n-Octadecanoate	$CH_3(CH_2)_{16}COO$-
20	0	Arachidate	n-Eicosanoate	$CH_3(CH_2)_{18}COO$-
22	0	Behenate	n-Docosanoate	$CH_3(CH_2)_{20}COO$-
24	0	Lignocerate	n-Tetracosanoate	$CH_3(CH_2)_{22}COO$-
14	1	Myristoleate	Δ^9-Tetradecenoate	$CH_3(CH_2)_3CH=CH(CH_2)_7COO$-
16	1	Palmitoleate	cis-Δ^9-Hexadecenoate	$CH_3(CH_2)_5CH=CH(CH_2)_7COO$-
18	1	Oleate	cis-Δ^9-Octadecenoate	$CH_3(CH_2)_7CH=CH(CH_2)_7COO$-
18	2	Linoleate	cis,cis-$\Delta^{9,}\Delta^{12}$-Octadecadienoate	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6CO$
18	3	Linolenate	all-cis-$\Delta^{9,}\Delta^{12},\Delta^{15}$-Octadecatrienoate	$CH_3CH_2(CH=CHCH_2)_3(CH_2)_6COO$-
18	3	Eleostearate	$\Delta^{9,}\Delta^{12},\Delta^{13}$-Octadecatrienoate	$CH_3(CH_2)_3(CH=CH)_3(CH_2)_7COO$-
20	4	Arachidonate	all-cis-$\Delta^{5,}\Delta^8,\Delta^{11},\Delta^{14}$-Octadecatrienoate	$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2CO$

Table 1.3 : Some common fatty acids.

The unsaturated fatty acids are named from the position of the double bonds in the structure: for example omega-6 or (n-6), omega-3 or (n-3) with the Greek letter ω or n

representing the last carbon atom in the chain, and the number representing the position of the carbon atom on which the first double bond is located in the chain from the last carbon atom; in the case of omega-3 or omega-6 the first double bond is found on the bond between the third and fourth carbon atom and on the bond between the sixth and seventh carbon atom from the methyl end respectively. Table 1.3 represents examples of fatty acids.

1.3.1.3 - Different classes of lipids

1.3.1.3.1 - Triglycerides

The synthesis of triglyceride is represented in Fig. 1.8 and is achieved by attachment of 3 fatty acid molecules to a molecule of glycerol by formation of ester bonds and removal of a molecule of water.

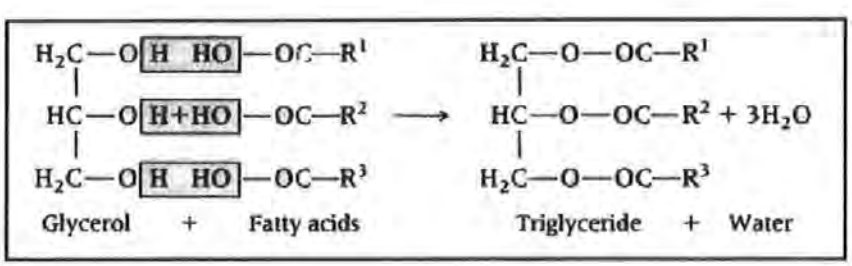


Fig. 1.8: Formation of a triglyceride molecule (from Stryer, 1995)

1.3.1.3.2 - Phospholipids and sphingolipids

In phospholipids one fatty acid of a triglyceride has been replaced by the inorganic compound phosphoric acid which leads to the formation of phosphatidic acid the basic component of phosphoglycerides. The common feature in membrane phospholipids is the linkage of a small hydrophilic alcohol to the phosphate by an ester bond leading to a variety of phospholipids according to the alcohol linked. Fig. 1.9 represents the structure of some common phosphoglycerides.

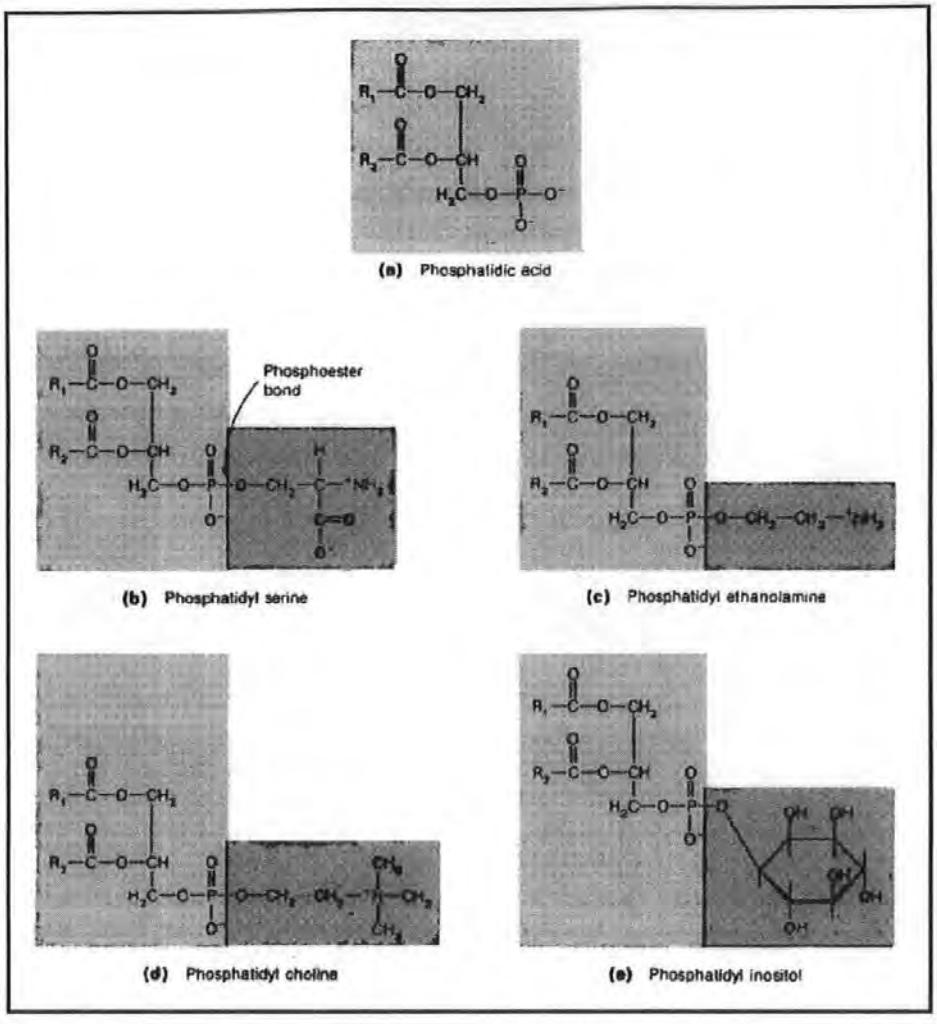


Fig. 1.9: Structure of some common phosphoglycerides (from Becker and Deamer, 1991)

Most of these alcohols contain an amino group charged at cellular pH and their juxtaposition to negatively charged phosphate makes the phosphoglycerides electrically neutral but highly polar in the head region. This highly polar head in turn juxtaposed to the two long non-polar chains (tail) of the phosphoglycerides gives the phosphoglycerides their amphiphatic nature that is so important to the membrane structure.

Sphingolipids are another class of lipids (Fig. 1.10) found in membranes of animal cells which are based not on glycerol but on the amine alcohol sphingosine. Through its amine group sphingosine can form an amide bond to a long chain fatty acid resulting in the formation of a ceramide. The hydroxyl group on the head of the molecule can accept a variety of polar groups leading to the formation of a whole family of sphingolipids.

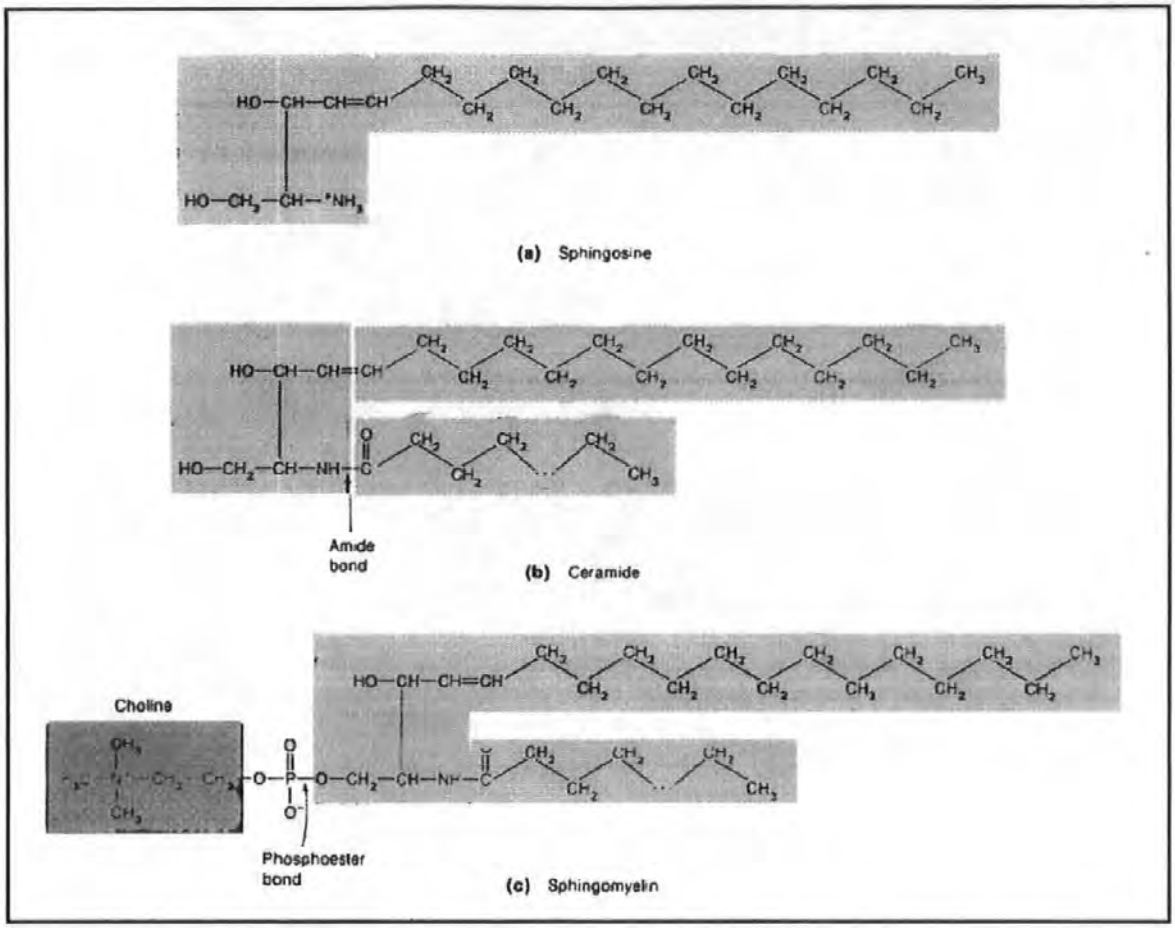


Fig. 1.10 : Structure of some common sphingolipids (from Becker and Deamer, 1991)

1.3.1.3.3 - Steroids

Steroids are lipids containing 4 joined rings and therefore differ greatly in their structure from the other categories. However they are very important molecules and play a variety of roles in the cells. Fig. 1.11 represents the structure of steroids

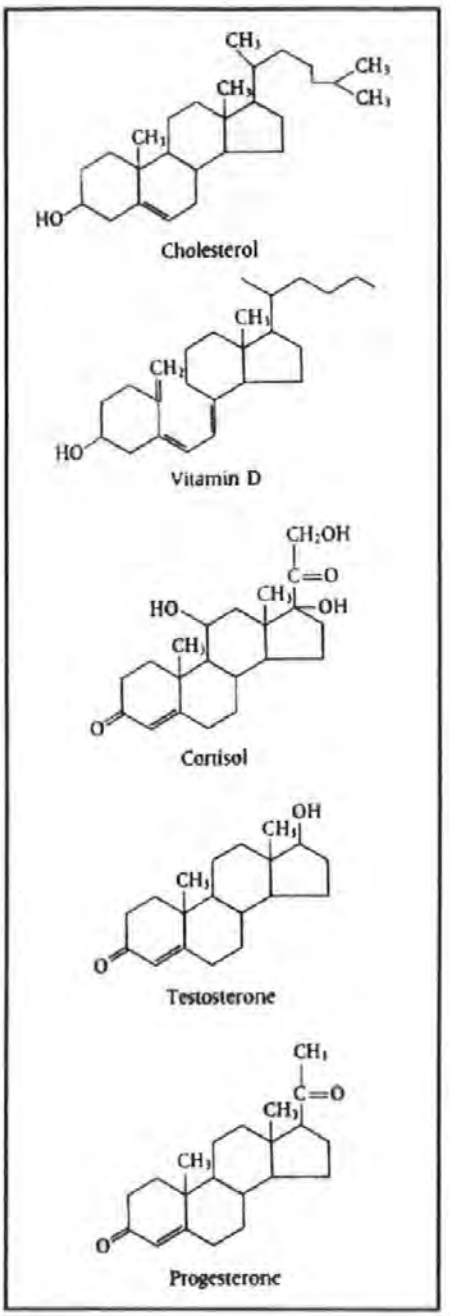


Fig. 1.11 : The structure of some biomolecules derived from steroid. (from Beck *et al.*, 1991)

1.3.1.4 - Sources

Different oils are known to be rich in different qualities of PUFAs. Olive oil is rich in (n-9) monounsaturated fatty acid, oleic acid, whereas corn oil or sunflower oil are rich in (n-6) PUFA linoleic acid. However fish oil such as cod liver oil are richer in (n-3) PUFAs such as eicosapentaenoic acid and docosahexaenoic acid. The fatty acid composition of various oils is presented in table 1.4 and the fatty acid composition of marine oils is presented in table 1.5.

Fatty acid	Composition (%)									
	Butter	*Tallow*	*Lard*	*Coconut oil*	*Olive oil*	*Corn oil*	*Soybean oil*	*Sunflower oil*	*Safflower oil*	*Menhaden oil*
8:0				5.6						
10:0	1			6.8						
12:0	2.5			52.0						
14:0	10.5	3.4	1.5	19.0						10.7
16:0	31.0	25.5	25.3	8.5	11.3	10.1	10.4	7.0	6.9	20.6
18:0	13.6	24.9	15.0	1.5	2.2	1.6	3.8	4.0	2.2	4.8
18:1 (n-9)	29.9	35.7	44.5	4.9	78.5	31.4	24.2	17.1	11.4	10.9
18:2 (n-6)	1.8	1.6	9.3	1.3	7.2	56.3	53.5	58.5	79.3	1.9
20:5 (n-3)										13.1
22:6 (n-3)										7.9

1.4 - Fatty acid composition of some common dietary fats and oils (from Beitz and Hansen, 1995)

Fatty acid	Composition (wt. %)					
	Capelin Jan Meyen	*Capelin Balsfjord*	*Sand eels Shetland*	*Herring Clyde*	*Cod liver*	*Mackerel*
14:0	7	8	7	7	5	3.5
16:0	13	30	12	13	12	19.1
16:1	10	8	5	7	12	7.6
18:0	1	1	1	1	2	3.8
18:1	14	23	6	10	23	26.2
18:2 (n-6)	2	2	1	1	1	-
18:3 (n-3)	1	1	1	1	1	2.6
18:4 (n-3)	4	4	4	3	2	2.4
20:1 (n-9)	16	4	13	13	8	3.1
20:5 (n-3)	8	11	11	6	13	9.2
22:1 (n-11)	15	4	19	23	5	3.1
22:6 (n-3)	6	8	9	6	11	12.7
Sat	21	39	20	21	19	26.4
Mono-unsaturated	55	39	43	53	48	40.0
(n-3) PUFA	19	24	25	16	27	26.9

1.5 - Fatty acid composition of fish oils (from Sargent and Henderson, 1993)

1.3.2 - Metabolism of lipids

1.3.2.1 - Biosynthesis of fatty acids

Fatty acids can be synthesised in the cytoplasm of cells where the first step begins with the assembly of malonyl CoA, a 3 carbon compound, by the carboxylation of acetyl CoA which serves as a primer. The 3 carbon compound malonyl CoA then reacts with another molecule of acetyl CoA and loses one carbon atom in the form of CO_2 while forming a 4 carbon compound named butyryl CoA. Butyryl CoA becomes the starting point of another similar addition of 2 carbons by the same process leading eventually to the formation of 16 to 18 carbon molecules.

Fig. 1.12 represents the overall reaction of synthesis of a fatty acid.

$$\text{AcetylCoA} + 7\ \text{MalonylCoA} + 14\ \text{NADPH} + 7\ H^+ \rightarrow CH_3(CH_2)_{14}COOH + 7\ CO_2 + 8\ \text{CoA} + 14\ NADP^+ + 6\ H_2O$$

Fig. 1.12: Biosynthesis of fatty acid

1.3.2.2 - Elongation, desaturation of fatty acids.

Although fatty acids can be synthesised by animal tissue, the ability of animals to desaturate fats remains limited and as a result some of the unsaturated fatty acids cannot be synthesised *de novo* and therefore must be supplied in the diet.

These polyunsaturated fatty acids, termed essential fatty acids, are linoleic acid [18:2 (n-6)] and α-linolenic acid [18:3 (n-3)], which serve as precursors of polyunsaturated fatty acids of the (n-6) and (n-3) family. In most animals double bonds can be introduced at the Δ^4, Δ^5, Δ^6, and Δ^9 positions counting from the carboxyl terminal but not beyond the Δ^9 position. In contrast, plants are able to introduce new double bonds at the Δ^6, Δ^9, Δ^{12} and Δ^{15} position, and can therefore synthesise the nutritionally essential fatty acids. The metabolic pathways with the precursor fatty acids and their long chain derivatives are presented in Fig. 1.13.

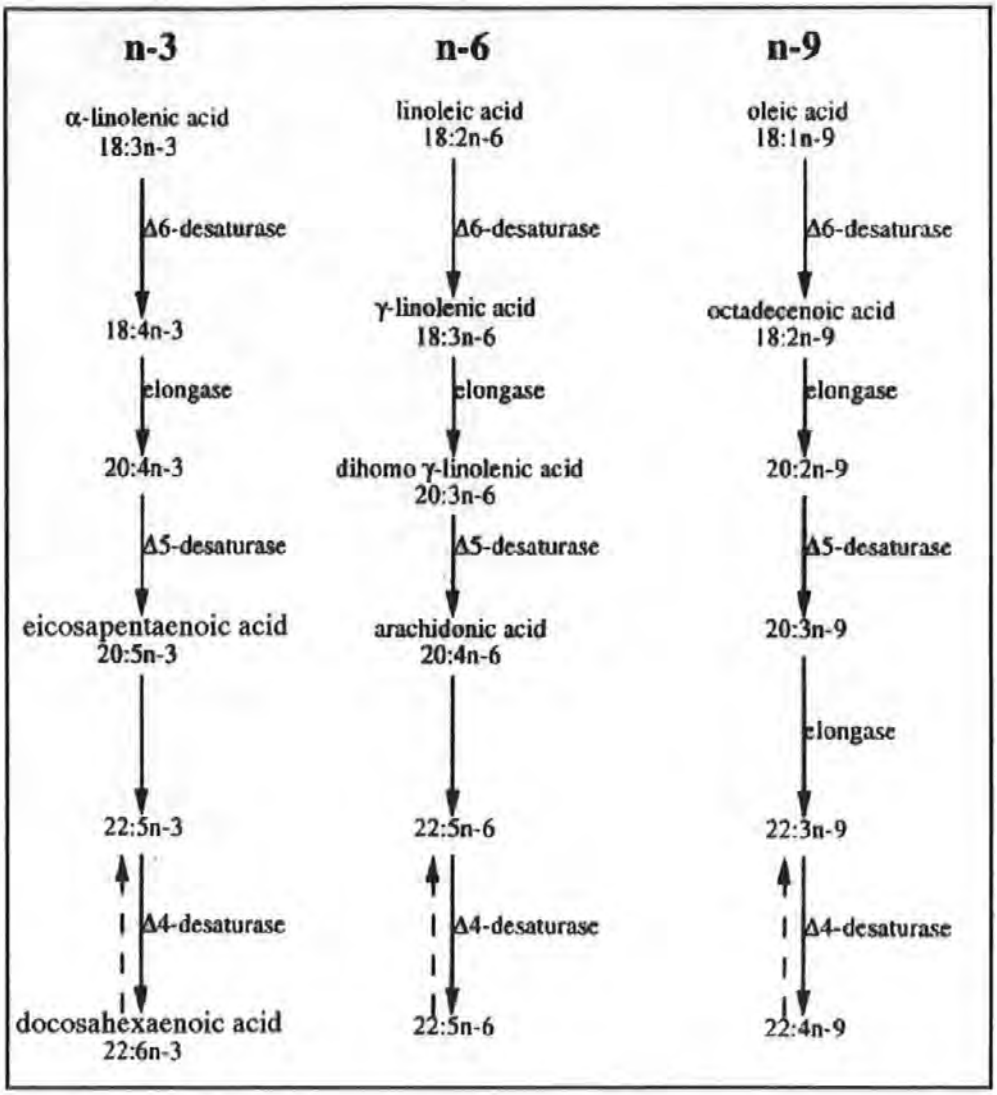


Fig. 1.13 : Main metabolic pathways for PUFAs synthesis and conversion (from Calder, 1993a)

However, the requirements for different PUFAs depends on the species considered. For instance, animals belonging to the cat family cannot synthesise arachidonic acid from linoleic acid, although other mammals seem to be able to perform this transformation. Therefore these animals have a specific requirement for arachidonic acid which can be then termed essential fatty acid.

In fish the liver is the major site for fatty acid synthesis and in contrast to mammals very little, if any, synthesis takes place in the adipose tissue (Greene and Selivonchick, 1987). Turbot, a carnivorous fish seems to be similar to the cat family, interestingly enough another carnivorous species. Indeed several investigations have demonstrated that turbot have poor abilities to convert any C_{18} fatty acids to longer chain fatty acids (Owen *et al.*, 1975; Cowey *et al.*, 1976; Gatesoupe *et al.*, 1977). Owen *et al.* (1975) did not observe any desaturation of dietary oleate, linoleate or linolenate and noticed that chain elongation of

these fatty acids was very limited when turbot were fed radioactively labelled fatty acids. Cowey *et al.* (1976b) confirmed this inability of turbot to desaturate C_{18} fatty acids. In a more recent investigation, Linares and Henderson (1991) using ^{14}C-labelled fatty acids showed that turbot were limited in their capacity to convert C_{18} PUFA to longer chain PUFA. They were however able to synthesise C_{22} PUFA from C_{20} PUFA.

It seems that with this deficiency in Δ5 desaturase and elongase activities, turbot are unable to synthesise arachidonic acid and eicosapentaenoic acid *de novo*, and therefore have a specific requirement for these two fatty acids in their diet.

1.3.2.3 - Digestion of lipids

In mammals, after being ingested, lipids are digested by the action of pancreatic lipases which break down the triglycerides to varying degrees into different molecules. The major products remain as free fatty acids, glycerols and monoglycerols. The lipase activity depends on the presence of bile, a complex fluid produced by the liver. Bile salts are derivatives of cholesterol which contain polar and non-polar regions and therefore are natural detergents. Bile salts are synthesised in the liver and are stored in the gall bladder before being released into the small intestine where they solubilise dietary lipids. They emulsify the lipids in the intestine, thus increasing the surface area of lipids, which first promotes their hydrolysis by lipase and secondly facilitates their absorption through the intestine.

After being absorbed, the products of fat digestion are picked up by the lymphatic system in the form of small globules of fat before being returned to the blood stream. The different lipids are transported in the body fluids by a series of lipoproteins classified according to increasing density. These lipoproteins consist of a core of hydrophobic lipids surrounded by polar lipids and a shell of apoproteins. These complexes can solubilise highly hydrophobic lipids and moreover their protein components contain signals that regulate the entry and exit of lipids at specific targets. Chylomicrons the largest of the lipoproteins transport dietary triacylglycerols, cholesterol and other lipids from the intestine to adipose tissue and the liver. The triacyl glycerols in chylomicrons are hydrolysed within a few minutes by lipases located in the capillaries of adipose and other

peripheral tissues. Very low density lipoproteins (VLDL) are primarily synthesised in the liver and deliver endogenously synthesised triacylglycerols to adipose tissue. The residue is transformed into low density lipo-proteins (LDLs) which are rich in cholesterol ester and whose role is to transport cholesterol to peripheral tissues. High density lipo-protein (HDL) synthesised by the liver transports cholesterol from tissues to the liver.

Different studies in fish have demonstrated the existence of lipase activity. Greene and Selivonchick (1987) in their review reported that various species were able to hydrolyse synthetic triacylglycerol or methyl esters. In most of the species though it seemed that saturated fatty acids were more resistant to hydrolysis than PUFAs. As pointed out by the authors, the fate of the free fatty acids after liberation from ingested triacylglycerol or wax ester was subject to debate. Whether most of the lipids are received as free fatty acids or whether long chain fatty acids are esterified into triacylglycerol in the epithelial cells leading to formation of VLDL-like particles is not clear.

A recent study by Koven *et al.* (1994), focused on the lipid digestion in turbot. The authors examined the lipid content and lipid composition of digesta from the stomach, foregut, hindgut and rectum of juvenile turbot. The data obtained suggests that a non-specific lipase is actively involved in lipid digestion in turbot particularly in the hindgut and rectum. The relatively low levels of free fatty acids (FFA) from the stomach digesta compared to the other segments reflects the low lipolytic activity of this organ. Some degree of preference for hydrolysis of PUFAs from triacylglycerols and specific absorption of PUFAs from the digesta was also observed, while saturated fatty acids and monoenes seemed more poorly adsorbed in the rectum.

1.3.2.4 - Oxidation in the cell

The initial event in the utilisation of fat as an energy source is the hydrolysis of triacylglycerol by lipases yielding a molecule of glycerol and three molecules of fatty acids. Then fatty acids can be broken down in the cytoplasm of cells to yield energy. The fatty acids are first converted to their CoA derivatives, acyl CoA, and then a stepwise removal of 2 carbon units in the form of acetyl CoA leads finally to complete oxidation of

triglyceride. A diagram presenting the pathway of fatty acid breakdown is shown in Fig. 1.14.

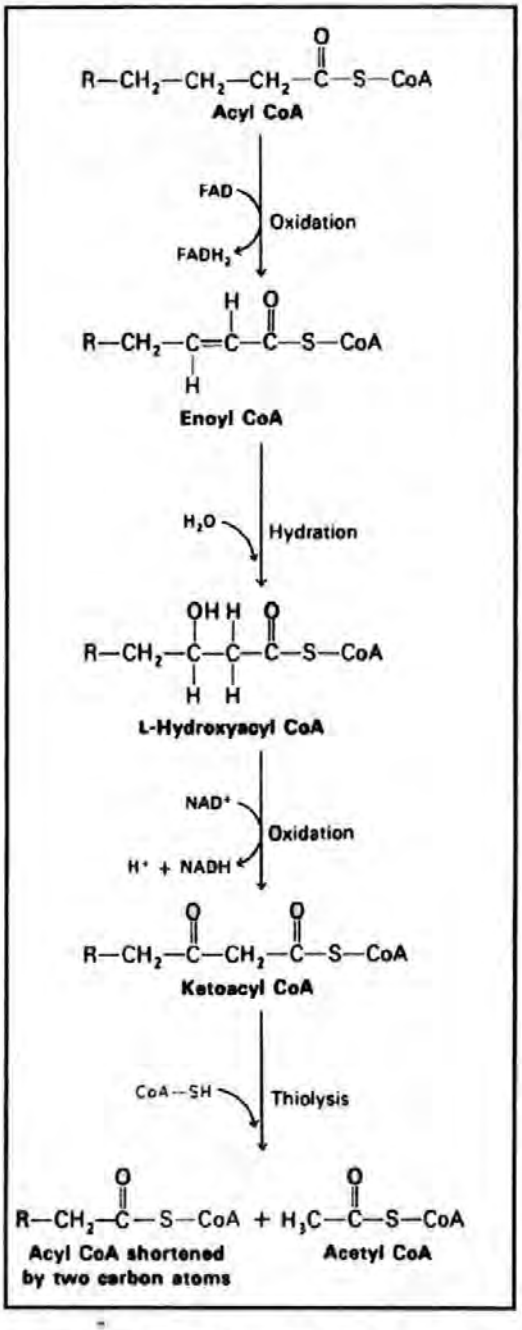


Fig. 1.14 : Sequence of events in the degradation of fatty acids (from Stryer, 1995)

A saturated acyl CoA is degraded by a recurring sequence of 4 reactions which are: oxidation linked to flavin adenine dinucleotide (FAD), hydration, oxidation linked to NAD^+ and finally thiolysis by HSCoA. The fatty acid is shortened by two carbon atoms, $FADH_2$ and NADH, and acetyl CoA are generated. NADH can then be oxidised by the respiratory chain where it yields 3 ATPs whereas $FADH_2$ yields 2 ATP molecules. For example, complete oxidation of palmitate will yield 129 ATP molecules.

The oxidation of unsaturated fatty acids is essentially similar to that of saturated fatty acids except for the necessary intervention of 2 supplementary enzymes: isomerase which converts a cis-double bond into trans-double bond, and epimerase which changes the configuration of an hydroxyl group.

1.3.3 - Biochemical functions of lipids

1.3.3.1 - Structural role of lipids

As discussed earlier lipids are very important constituents and have three major functions: first they are building blocks of phospholipids in biological membranes, secondly some serve as hormones and intracellular messengers but finally they are important energy sources.

1.3.3.1.1 - Triglycerides

The primary purpose of triglycerides in the cell is to store energy. These molecules are highly reduced and anhydrous which gives them their ability to store and concentrate energy. The complete oxidation of fatty acids yields to 9 kcal/g in contrast with about 4 kcal/g for carbohydrates and proteins.

1.3.3.1.2 - Phospholipids and sphingolipids

Phospholipids exhibit similarities with triglycerides but differ in their role and are critical to the bilayer structure found in membranes.

The highly polar head, juxtaposed to the two long non polar chains (tail) of the phosphoglycerides gives them their amphiphatic nature which is crucial to the membrane structure.

Fig.1.15 represents the amphiphatic nature of membrane phospholipids and their disposition in the cell membrane.

Sphingolipids are another class of lipids found in membranes of animal cells.

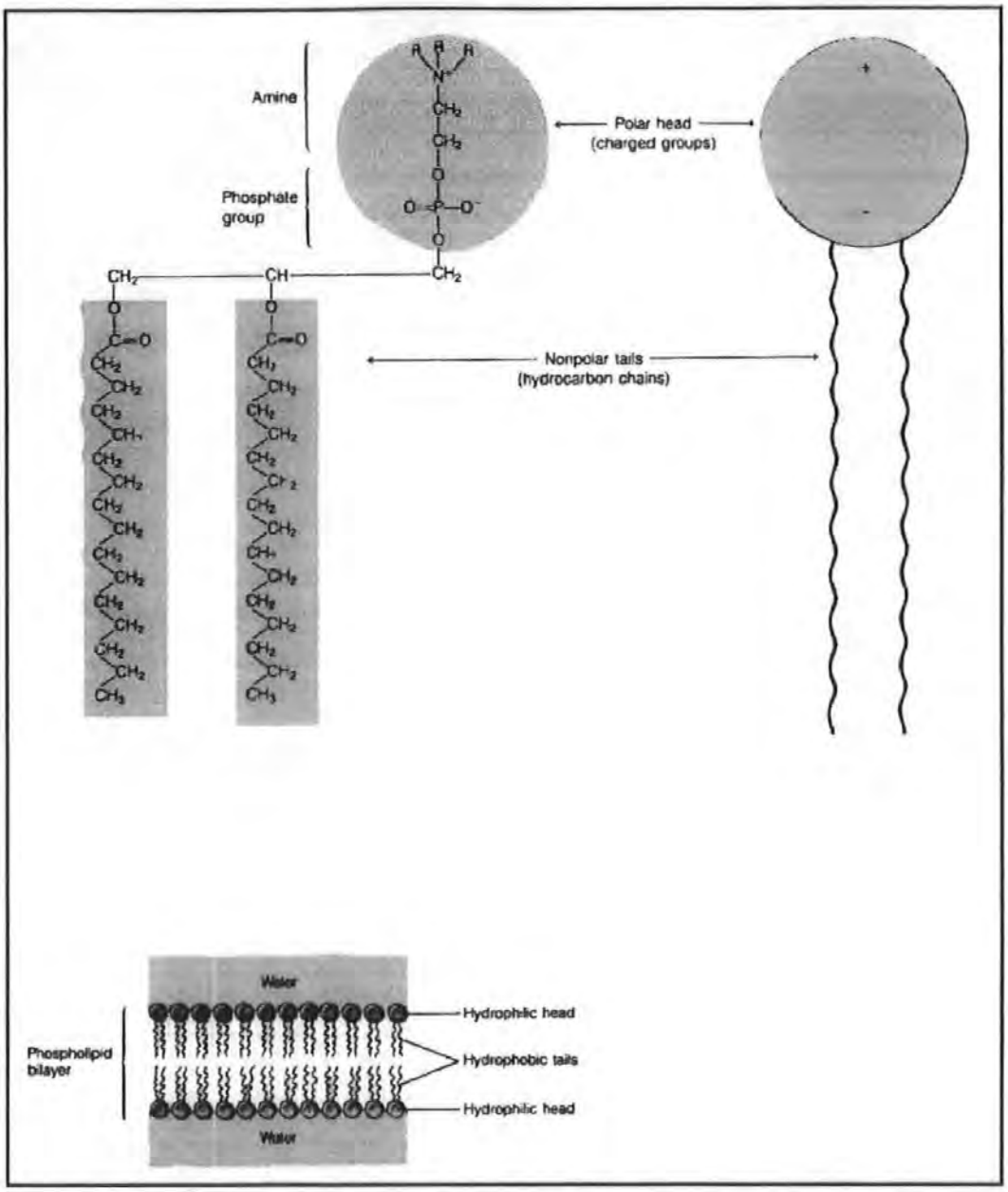


Fig. 1.15 : Membrane phospholipids and their disposition in the membrane (form Becker and Deamer, 1991)

1.3.3.1.3 - Steroids

Steroids differ greatly from the other categories of lipids by their structure but their role in metabolism remains essential.

Cholesterol is a component of all eucaryotic plasma membranes with the exception of the inner membranes of mitochondria, where it is absent. It is essential for growth and viability of cells in higher organisms. The molar ratio of cholesterol to phospholipids is a major determinant of membrane fluidity or microviscosity (Johnston, 1988).

1.3.3.2 - Lipids as precursors of messengers

Some lipids can be precursors of very important molecules for cell physiological processes. For instance C_{20} PUFAs can act as precursors for eicosanoid synthesis which will be reviewed in the next section for their role in immunomodulation. Other lipids are able to generate secondary messengers which are used by the cells to activate or regulate different physiological processes which largely go beyond immune function. This is the case of phosphatidylinositol 4,5 biphosphate (PIP_2) a phosphoinositide within the cell membrane. The mechanism of action is presented in Fig.1.16.

This phospholipid can be broken down in two secondary messengers when a neighbouring receptor (R) is activated by binding of its specific ligand (L) to the outer surface of the plasma membrane (a). The receptor-ligand complex associates with the G protein (called Gp), causing the displacement of GDP by GTP and the dissociation from the G protein of the α-subunit-GTP complex. The α-subunit-GTP complex then binds tightly to a molecule of phospholipase C (P), activating it for cleavage into one molecule of inositol triphosphate (IP_3) and diacylglycerol (DAG), the second messengers (b).

IP_3 is released in the cytoplasm where it activates the release of calcium from intracellular stores (c), whereas DAG remains in the membrane but in collaboration with the calcium release, activates a protein kinase C which in turn activates many enzymes (d). Thus DAG and IP_3 serve as messengers capable of mediating the wide variety of regulatory extracellular signals. This mechanism is involved in the regulation of many cell functions such as platelet activation, muscle contraction, insulin secretion, amylase secretion and glycogen degradation.

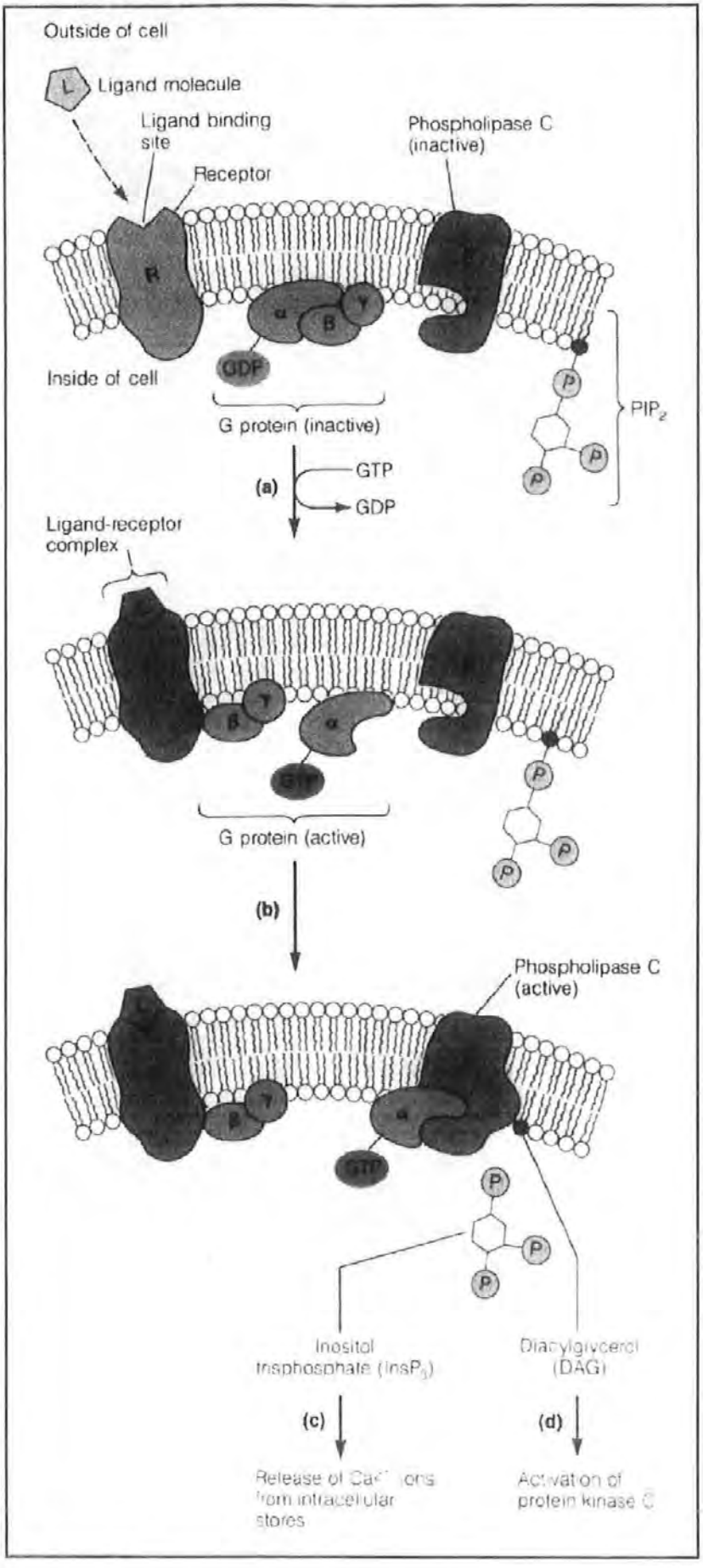


Fig. 1.16 : Mechanism of action of IP_3 and DAG as second messengers (From Becker and Deamer, 1991)

1.3.3.3 - Steroid hormones

Except for its structural role in membranes cholesterol is a key molecule in metabolism as it is the precursor of a variety of molecules involved in various metabolic pathways.
Bile salts, as seen earlier, are polar derivatives of cholesterol and are essential to lipid digestion.
Cholesterol is also the precursor of 5 different classes of steroid hormones and in that respect plays a fundamental role in metabolism. These classes include progestagens, glucocorticoids, mineralocorticoids, androgens and oestrogens. Progesterone, a progestagen, is essential for the maintenance of pregnancy. Androgens are responsible for the development of male secondary sex characteristics whereas estrogens are responsible for the developments of female ones.
Glucocorticoids, including hormones such as cortisol, are involved in the regulation of gluconeogenesis and the degradation of fat and protein. Mineralocorticoids increase the reabsorption of salts by the kidney into the blood and are therefore essential in the maintenance of blood volume and pressure.
Finally vitamin D3 (cholecalciferol), which plays an essential role in the metabolism of calcium and phosphorus, is also derived from cholesterol.

1.3.4 - Lipids and immune and haemostatic functions

1.3.4.1 - Greenland eskimos

The importance of dietary lipids in the development of certain diseases was noticed in the 1970's in Greenland eskimo populations where different studies showed a lower incidence of ischaemic heart diseases or other thrombotic diseases. Thrombosis is a condition where an unwanted clot occurs within the blood stream, either in an artery or a vein and as a result an obstruction of blood flow occurs which can be fatal. This represents an important issue in "westernised" populations as a large proportion of deaths are caused by thrombosis occurring in critical locations in the body. For instance thrombosis in the coronary artery,

can result in a myocardial infarction, and thrombosis in a branch of pulmonary artery may cause a pulmonary embolism.

Several expeditions were organised by Bang and Dyeberg (1980) to the Greenland eskimos in order to investigate the blood lipid levels of eskimos and development of ischemic heart diseases and other artherosclerotic conditions believed to be linked to blood lipid levels. This first study carried out in 1970 revealed that compared to Danes, eskimos have lower plasma levels of total lipids, cholesterol and triglycerides. The fatty acid composition of plasma lipids was also analysed and it was revealed that essentially the fatty acids of the (n-6) family in eskimos were replaced by (n-3) fatty acids. The more noticeable difference concerned linoleic [18:2 (n-6)] and arachidonic acid (AA) [20:4 (n-6)] which were much lower in Greenlanders, whereas eicosapentaenoic (EPA) [20:5 (n-3)] acid was found to be much higher in this population.

Blood lipid level is influenced by diet so further expeditions were organised to analyse the lipid content of Greenlander's food and showed that the food lipid composition grossly reflected that of the plasma lipids. The eskimo consumption of linolenic [18:3 (n-3)] and linoleic acid [18:2 (n-6)], considered to be the precursor in western food of AA [20:4 (n-6)], was found to be much lower than that of the Danes. Another remarkable point was the higher intake of long chain polyunsaturated fatty acids of the (n-3) family by eskimos, in particular EPA [20:5 (n-3)], docosapentaenoic acid [22:5 (n-3)], and DHA [22:6 (n-3)], compared with Danes whose intake of PUFAs was dominated by fatty acids of the (n-6) family.

Following this expedition a fourth study was undertaken by the same authors in 1978 to investigate the haemostatic function of eskimos. The results from that study showed that eskimos had a much slower clotting time than Danes and that the mechanism of antithrombotic tendency in the Greenlanders was due to a substantially decreased platelet aggregability, causing a reduced inclination in thrombus formation and therefore a reduced incidence of thrombotic diseases. Bang and Dyeberg (1980) hypothesised a shift in the balance of pro and anti-aggregatory prostaglandins (PGs) toward anti-aggregation to explain this decrease in platelet aggregability. This shift was caused by a high level of EPA [20:5 (n-3)] acid in structural lipids and a low level of AA [20:4 (n-6)] induced by the composition of dietary fat. Indeed these two PUFAs can act as precursors of PGs but in

different ways. AA leads to the formation of PGs_2 in vessel walls which has an anti-aggregatory role but also to the formation of thromboxane by platelets which have a proaggregatory role. On the other hand EPA leads to the formation of PGs of the 3 family with an anti-aggregatory role but does not lead to formation of any proaggregatory factor, therefore inducing a longer bleeding time before coagulation.

1.3.4.2 - Lipids as precursors of eicosanoids

Other studies on mammals in the 1980's revealed that dietary variations in lipid composition could influence the production of different prostaglandins or leukotrienes by cells of the immune system. Lee *et al.* (1985) showed that seven normal subjects fed with diets supplemented in EPA and DHA for 6 weeks saw their production of leukotriene B_4 inhibited in their neutrophils and monocytes. A similar study on rats resulted in the same conclusion that an EPA rich diet could decrease the accumulation of leucocytes at sites of inflammation via its modulatory role on the production of lipoxygenase products, especially the LTB_5/LTB_4 ratio (Terano *et al.*, 1984). Leukotriene B_5, the 5-lipoxygenase product of eicosapentaenoic acid was synthesised by human polymorphonuclear leucocytes from endogenous eicosapentaenoic acid incorporated into cellular phospholipids during dietary supplementation (Strasser *et al.*, 1985).

1.3.4.2.1 - Biosynthesis of eicosanoids.

As the Greenland eskimo study suggested the modulation role of dietary lipids in the incidence of ischemic heart disease and thrombosis seemed to be played by prostaglandins, molecules issued from the metabolism of C_{20} PUFAs. Prostaglandins are part of a group of compounds named eicosanoids, derived from the oxygenation of C_{20} PUFAs released from membrane phospholipids, which are believed to play an important role in the modulation of the immune response. The production pathways of eicosanoids have been described by numerous authors although figures will be presented here from the review of Smith and Fitzpatrick (1996).

Eicosanoids are made of three families, prostanoids, leukotrienes and lipoxins, and epoxyeicosatrienoic acid. These three groups of compounds are synthesised via three

different pathways, the cyclooxygenase, the lipoxygenase and the epoxygenase pathways. These routes are presented in Fig. 1.17.

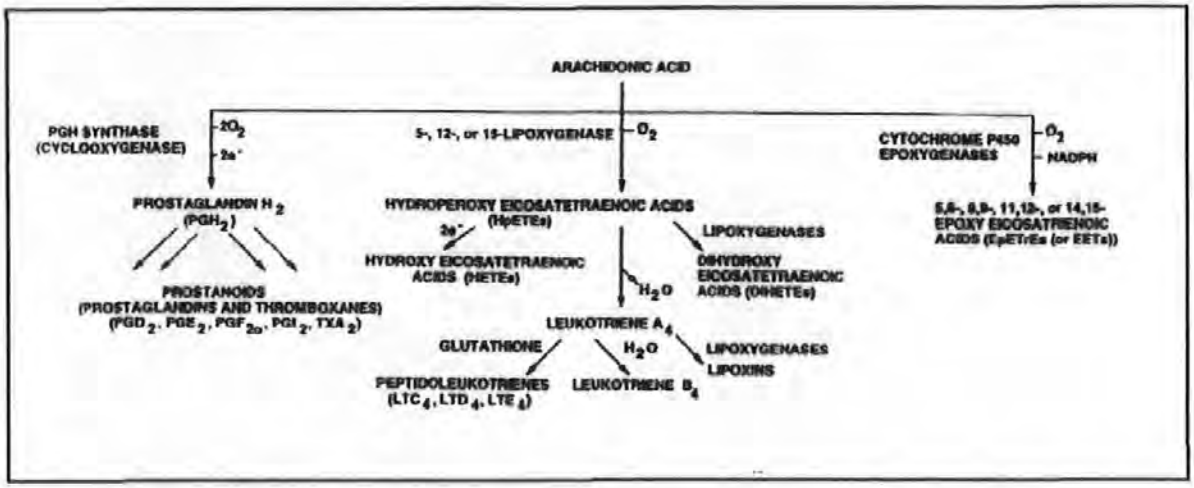


Fig. 1.17: Biosynthesis of eicosanoids from arachidonic acid. (from Smith and Fitzpatrick, 1996)

The common first step is the liberation of AA from membrane phospholipids or other precursors through phospholipase action.

The cyclooxygenase pathway leads to the formation of prostanoids which are composed of prostaglandins (PG) and thromboxanes (TX). PGs and TXs of the 2 series are the most well known, although it is important to note that two other series of PGs and TXs exist. Series 1 is generated from the metabolism of 20:3 (n-6) while series 3 is generated from the metabolism of 20:5 (n-3). The second step consists in the conversion of arachidonate to the prostaglandin endoperoxides PGH_2 followed by a third cell-specific step which leads to the conversion of PGH_2 to one of the major prostanoids.

In addition to the cyclooxygenase pathway, AA and other eicosanoids precursors may enter the lipoxygenase pathway presented. This pathway leads first to the formation of hydroperoxy eicosatetraenoic acids (HpETEs) which in turn are transformed into leukotrienes (LT) through the unstable 5,6-epoxide intermediate, LTA_4, or to lipoxins (LX). There also the different precursors will lead to different LT families with 20:3 (n-6) leading to the 3-series while 20:4 (n-6) and 20:5 (n-3) generate respectively the 4- and 5-series of leukotrienes.

Finally the epoxygenase pathway leads to formation of epoxy eicosatrienoic acids by introduction of a single O_2 atom by cytochrome P450. This products although having a

wide range of biological activities, do not play a role in immune system and will be ignored in the next section.

1.3.4.2.2 - Sites of production of eicosanoids.

Eicosanoids generated from the metabolism of phospholipids released from the cell membrane can be produced at various sites in the body.

Johnston (1988) underlines the controversy which exists about whether macrophages are the only cells able to produce cyclooxygenase products or whether lymphocytes are also producers of these molecules. However, there was evidence to indicate a lack of cyclooxygenase activity in lymphocytes but a definite one in macrophages in *in vitro* studies. On the other hand, many cells of the immune system such as neutrophils, macrophages, mast cells, basophilic leukaemia cells, seem to demonstrate lipoxygenase activity although mononuclear phagocytes are, in general, established as producers of LT. But Johnston (1988) also points out some contrast in different findings whether lymphocytes can metabolise 20:4 (n-6) to lipoxygenase products. However, in mammals, recent research using lymphoblastic B- and T-cell lines and molecular probes to localise lipoxygenase and 5-lipoxygenase activating protein (shown to be essential for leukotriene biosynthesis) have settled the debate. 5-LO products can be generated by lysates of B but not T-lymphocytes or by intact B lymphocytes co-incubated with ionophore and glutathione depleted agents (Claesson *et al.*, 1992, Jacobsson *et al.*, 1992).

1.3.4.3 - Eicosanoids in fish

The mechanisms of eicosanoid biosynthesis in fish have been reviewed by Rowley *et al.* (1995). They are essentially the same as the ones described for mammals although it is worth noting that 22:6 (n-3) has also been described as a possible precursor for prostaglandin synthesis in fish (Bell *et al.*, 1986).

Studies in marine animals seem to indicate that different tissues are able to produce eicosanoids. Ogata *et al.* (1978) showed that tissue homogenates of sea-squirt (*Halocynthia roretzi*), crab (*Patinopecten yessoenssis*), hard clam (*Meretrix lusoria*) and carp (*Cyprinus carpio*) were capable of prostaglandin biosynthesis when incubated with

^{14}C-labelled dihomo-γ-linolenic acid. This biosynthesis activity was thereafter more specifically studied in selective tissues of various fish. Anderson *et al.* (1981) investigated prostaglandin biosynthesis in the skin of plaice (*Pleuronectes platessa* L.). They characterised the synthesis of a material from AA which had the properties of PGE_2. They found that the PG synthetase activity of the skin was confined almost exclusively to the fraction of plaice skin that corresponded to the microsomal fraction of mammalian cells, and this activity was inhibited when preincubation of the microsomal enzyme with EPA was performed. Tissue homogenates from turbot (*Scophthalmus maximus*) were also the subject of investigations for prostaglandin biosynthesis. Henderson *et al.* (1985) measured the conversion of ^{14}C-labelled AA and EPA from homogenates of gills, intestine and liver. The higher conversion into PGs was obtained from gill homogenates and an apparent competition between AA and EPA as precursors of PGs was also noted: in the presence of equimolar concentrations of EPA the incorporation of AAs into PGs was decreased. Recent studies with juvenile turbot focused on feeding different qualities of PUFAs for 11 or 12 weeks and measuring the subsequent production of prostaglandins by various tissue homogenates (Bell *et al.*, 1995a and 1995c). The first one unfortunately only measured the levels of PGE_2 and $PGF_{1\alpha}$ after feeding the fish with different ratios of AA:DHA [docosahexaenoic, 22:6 (n-3)]. Nevertheless, this study concluded that fish fed the lowest dietary levels of AA had reduced levels of prostaglandins in their tissue homogenates and that dietary lipid composition was influencing the fatty acid composition of phospholipids of such fish.

The other study focused on dietary supplementation with EPA and GLA and its influence on production of PGE_1, PGE_2 and PGE_3 by juvenile turbot. The inability of turbot to desaturate dihomo γ-linolenic acid (DHGLA) to AA but the high elongase activity allowing selective accumulation of DHGLA [20:3 (n-6)] from GLA [18:3 (n-3)] in the tissues was used in this study to investigate the modulatory role of these precursors upon prostaglandin production. These dietary treatments resulted in the accumulation of either DHGLA or EPA and AA according to the feeding regime. This greatly influenced the qualities of prostaglandins measured in tissue homogenates with PGE_1 found in greater quantities in almost all tissues examined in fish fed with GLA diets (borage oil diets) compared to fish fed with EPA diets (marine oil diets). The ratio of PGE_2/PGE_1 was

significantly affected in all tissue homogenates and found to be much higher in tissues sampled from marine oil fed fish (Bell *et al.*, 1995c). It is important to note these ratios in prostaglandin production vary as it is established that the 1- and 3- series of PGs have lower activity compared to their AA-derived homologue PGs of the 2-series. A change in such a ratio could therefore influence the immune response.

Other studies, focused on immunity, investigated the ability of peculiar cells involved in the immune response to synthesise eicosanoids. Pettitt *et al.* (1991) reported the ability of rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages maintained in short term culture to synthesise lipoxins and leukotrienes when incubated with A23187 or opsonised zymosan. Trout macrophages were shown to contain both 5- and 12-lipoxygenases as judged by the presence of 5- and 12-HETEs in supernatants of challenged cells. Although LXA_4 could be detected there were no detectable amounts of LXB_4 in the supernatants indicating an independence in the big synthetic pathways of these two lipoxins.

The production of eicosanoids by different types of cells in fish has been reviewed by Rowley *et al.* (1995). Different studies suggest that monocytes/macrophages, granulocytes, and thrombocytes all have a significant eicosanoid-generating capacity in fish. Once again the question whether lymphocytes are able to produce eicosanoids arises. The possession of 12-LO activity in fish lymphocytes has been demonstrated although it is uncertain whether fish lymphocytes possess 5-LO activity. A study by Secombes *et al.* (1994) suggests though that T lymphocytes possess a cyclooxygenase pathway, although no LO products could be detected in the supernatant of cell suspensions.

1.3.4.4 - Immunomodulatory and haemostatic roles of eicosanoids

Many studies in the 1980's focused on the immunomodulatory role of lipids and have tried to explain the role of eicosanoids on the immune system in mammals. As was correctly pointed out by Johnston in her review (1988) the task is very complex due to the interactions of cells within the immune system : *"lymphocytes such as T-cells, and macrophages are interdependent in that T-cells may produce 20:4 (n-6) for macrophages to produce PG, which in turn may regulate the T-cell production of lymphokines. Another possibility is that T cell-produced lymphokine stimulates macrophage PG production,*

which then regulates further responses". It is then clear that any attempt to measure *in vivo* production of cyclooxygenase or lipooxygenase products will only produce information on the overall production resulting from the interaction of different cells and probably from a succession of different activation steps in the immune response. Besides the bioactives PGs are rapidly converted to inactive metabolites and therefore the non-detection of such compounds at one point in time does not mean that they have not been produced earlier on and played a key role in the processes of immune response before being transformed to inactive and undetectable metabolites.

All these difficulties lead us towards employing *in vitro* studies aiming to either add known quantities of exogenous eicosanoids to different cell populations or add inhibitors of cyclooxygenase and/or lipoxygenase in the medium of culture and measure the subsequent effects on various activities of these cell populations. The investigation of how eicosanoids affect individual cell populations seems essential before one attempts an explanation and integration of the effects of eicosanoids in the complex succession of events occurring during the immune response at the organism level.

The effect of eicosanoids on different cell populations in fish has been the subject of a few studies. Knight *et al.* (1993) showed that PGE_2 and 12-HETE enhanced the *in vitro* phagocytosis of yeast test particles by trout (*Oncorhynchus mykiss*) macrophages whereas LXA_4 and LTB_4 had no demonstrable effect. They further demonstrated that these effects were independent of intracelular calcium concentrations. Lloyd-Evans *et al.* (1994) also showed that some of the eicosanoids released during activation of trout thrombocytes played a role in the aggregatory behaviour of thrombocytes. LXA_4 and LTB_4 had aggregatory potential at high concentrations (10 μM) whereas 12-HETE had no significant effect. Secombes *et al.* (1994) showed that T-lymphocytes taken from the head kidney of rainbow trout and stimulated by phytohaemagglutinin-P (PHA-P) for proliferation were generating prostaglandins whereas no leukotrienes or lipoxins above basal level could be detected in the supernatant of stimulated cell suspensions. However, when adding prostaglandins or lipoxins to the culture medium, cell proliferation was inhibited and when adding leukotrienes, the proliferation was stimulated. The authors concluded that this might indicate that leukotrienes or lipoxins are generated via indirect mechanisms, suggesting in rainbow trout a complexity in the interaction of cells in the immune system

and the release of mediators during the immune response. Other studies on fish have also been reviewed by Rowley *et al.* (1995). One studies on winter flounder (*Pseudopleuronectes americanus*) (Laudan *et al.*, 1986) and the other in rainbow trout (Rainger *et al.* 1992), illustrated the drawback in using inhibitors of COX or LO *in vivo* which does not allow clear targeting of these compounds inside the individual. However, Laudan *et al.* (1986) concluded that PGs were involved in immunosuppressive activity of a parasite whereas Rainger *et al.* (1992) were unable to demonstrate a clear effect of indomethacin, a COX inhibitor, on antibody generation in trout, as its action varied from inhibition to stimulation according to the dose administered. However, a dose dependent inhibitory effect of nordihydroguaiaretic acid (NDGA), a LO inhibitor, on antibody synthesis against *Aeromonas salmonicida* was observed (Rainger *et al.*, 1992).

Further studies were undertaken and administration of a stable analogue of PGE was used instead of inhibitors of COX or LO generation. A clear immunosuppressive action of 16,16-dimethyl PGE_2 on the generation of plaque forming cells (PFC) to sheep red blood cells and specific antibody to *Aeromonas salmonicida* were observed in that study (Knight & Rowley, 1995). Cultures of trout splenic leucocytes with SRBC in the presence of PGE_2, PGE_3 or 16,16-dimethyl PGE_2 showed a dose dependent inhibition of PFC, while the LO-products were without clear effect.

It seems premature to summarise the action of eicosanoids on different cell populations in fish as it appears the same compound can have different effects on different cell populations or on different cell functions. Feeding different dietary lipids seems to influence the production of eicosanoids by fish tissues and might therefore induce changes in the immune response but any attempt to explain the underlying mechanisms of these modulations is still in its infancy and must be considered with care. Therefore, it is interesting to examine in a wider context what the effects of lipid nutrition are on fish before considering the subtle mechanisms underlying these changes.

1.3.4.5 - Lipid nutrition in fish

In fish studies have focused on the influence of dietary supplementation with different oil qualities upon growth, fatty acid composition of different organs and on the effect of such

supplementation upon some immune parameters. Generally preparations of diet with fish oil is quite common practice in aquaculture, as fish oil, such as cod liver oil, provides essential fatty acids (EFA) resulting in good growth and feed conversion and a good energy source resulting in sparing action on dietary protein. Such oils are rich in (n-3) PUFAs which have been shown (see above) to modulate eicosanoid synthesis in mammals. The plaice (*Pleuronectes platessa*) was one of the first fish studied in an investigation on the influence of dietary lipids on tissue fatty acid composition (Owen *et al.*, 1972). This study revealed that in fish offered a diet containing corn oil and cod lipid, the ratio of unsaturated fatty acids in the tissues resembles that of the diet. The triglycerides of fish maintained on a fat free diet were depleted of essentially all fatty-acid classes. Moreover, the inclusion of 1% of unsaturated fatty acids in a diet together with 7% saturated fat resulted in enhanced uptake of all the dietary fatty acids. Rainbow trout (*Oncorynchus mykiss*) fed with diets containing no polyunsaturated fatty acids showed poor growth and feed conversion; furthermore linolenic acid (18:3 (n-3)) was shown to have an essential role in trout (Castell *et al.*, 1972). Watanabe *et al.* (1974) came to the same conclusions when studying the effect of dietary methyl linolenate on growth of rainbow trout. Takeushi and Watanabe (1977) showed that both 20:5 (n-3) and 22:6 (n-3) have an EFA efficiency higher than that of 18:3(n-3) in rainbow trout. Watanabe *et al.* (1975a) produced a series of studies where they investigated the effect of methyl linoleate and linolenate on growth of carp (*Cyprinus carpio*). EFA requirement of carp was found to be much lower than that of rainbow trout, and carp could grow without abnormality for a fairly long period without EFAs, even though feeding a fat-free diet resulted in the lowest growth. A further study showed, nevertheless, that carp required not only linoleic but also linolenic acid and that fish fed diets containing fatty acids of both (n-6) and (n-3) types showed good growth condition (Watanabe *et al.*, 1975b).

Turbot (*Scophthalmus maximus*), have been the subject of numerous studies concerning fatty acid requirements and the influence of various fatty acid feeding regimes on growth and on the fatty acid composition of different organs (Cowey *et al.*, 1976 a,b; Gatesoupe *et al.*, 1977a,b; Léger *et al.*, 1979; Bell *et al.*, 1985a,b; Tocher and Sargent, 1987; Linares and Henderson, 1991; Castell *et al.*, 1994; Bell *et al.*, 1995a,b,c). Cowey *et al.* (1976a,b) were the first to demonstrate that arachidonic acid was inferior to PUFAs of the (n-3)

series in maintaining growth rates in turbot. Moreover, the results did not suggest any evidence for desaturation of dietary oleic acid, linoleic acid or linolenic acid by the turbot. In an initial study Gatesoupe *et al.* (1977a) demonstrated the necessary supplementation of diet by (n-3) PUFAs of turbot. In a second study the supplementation of diets with PUFAs of the series (n-3) were shown to have a positive effect on growth of turbot compared to supplementation with PUFAs of (n–9) series which depressed growth (Gatesoupe *et al.*, 1977b). In a subsequent study, Léger *et al.* (1979) presented evidence indicating the absence of elongase and desaturase activity in turbot, leaving these fish unable to elongate 18:2 (n-6) present in the diet into 20:4 (n-6) and therefore making them dependent on external sources of (n-6) HUFAs. Watanabe in his review (1982) underlined the fact that turbot were unable to convert dietary 18:2 (n–6) to 20:4 (n–6) when fed with corn oil or endogenous 18:1 (n–9) to 20:3 (n–9) when fed EFA deficient diet. Therefore, although turbot appeared to have an EFA requirement for fatty acids contained in cod liver oil, the requirement for fatty acids was not satisfied by 18:3 (n–3) and although positive growth was found when fed 18:3 (n–3) C_{20} and C_{22} were still more effective (Gatesoupe *et al.*, 1977b).

In a series of studies, Bell *et al.* (1985a) showed that although there are relatively constant PUFA levels in the phospholipids from the fish maintained on different regimes of PUFA, there were marked differences in the composition of these PUFAs showing that turbot are unable to maintain a constant chain length and desaturation of the PUFA in the face of dietary changes. Therefore, they require a correct balance of the three main PUFAs, 20:4 (n-6), 20:5 (n-3), and 22:6 (n-3). When fed for 16 weeks with a diet deficient in PUFA or deficient in (n-3) PUFA many of the fish died or showed depressed growth (Bell *et al.*, 1985b). Castell *et al.* (1994) showed that best survival rates were obtained for juvenile turbot fed with arachidonic acid (AA, 20:4 (n-6)) supplementation compared to other dietary treatments with docosahexaenoic acid (DHA, 22:6 (n-3)).

1.3.4.6 - Lipids and immune function in fish.

A few studies focused on immunomodulation by lipids in fish. Rainbow trout (*Oncorhynchus mykiss*) was studied for the influence of saturated and polyunsaturated

fatty acids on immune parameters (Ashton *et al.*, 1994; Kiron *et al.*, 1995). Supernatants of ionophore challenged head kidney leucocytes of rainbow trout fed with various diets were tested for their ability to chemoattract neutrophils by Ashton *et al.* (1994). The migration of leucocytes was found to be increased when using leucocyte supernatants from fish fed diets containing fosol (a fish oil) or menhaden oil compared to supernatants from fish fed with a diet containing sunflower oil (Ashton *et al.*, 1994). *In vitro* killing of bacteria by macrophages and antibody production were compromised by dietary essential fatty acid deficiency in fish (Kiron *et al.*, 1995). PUFA-fed fish were stronger in resisting pathogens but excess levels of (n-3) highly unsaturated fatty acids seemed to increase the mortality percentage of fish exposed to infectious haematopoietic necrosis virus (IHNV) pathogenic challenge compared to fish fed with linoleic or linolenic acid (Kiron *et al.*, 1995). Bowden *et al.* (1994) showed that dietary cholesterol reduced the phagocytic ability of trout macrophages to ingest foreign erythrocytes. The authors explained this reduction by the ordering effect of cholesterol on membranes and the consequent reduction in membrane fluidity by cholesterol.

Salhi *et al.* (1994) showed that diets rich in (n-3) HUFAs supported the best survival rates of larval gilthead seabream (*Sparus aurata*). Atlantic salmon (*Salmo salar*) were fed on diets containing either fish oil or sunflower oil therefore varying the ratio of (n-3)/(n-6) PUFA from 5.2 to 0.3 respectively. Fish were fed for a period of 150 days or 112 days on the different regimes but none of the non-specific immune parameters measured showed any significant differences. However, fish fed on the high ratio showed a significantly higher number of B cells responding to *Aeromonas salmonicida* after vaccination suggesting that Atlantic salmon fed with a low ratio of (n-3)/(n-6) PUFA may be less resistant to infection (Thompson *et al.*, 1996). Another study on Atlantic salmon by Bell *et al.* (1996), indicates that no significant differences were noticed in haematocrit, serum protein concentration, complement activity, lysozyme concentration or anti-protease activity for fish fed for 12 weeks with diets containing either fosol, sunflower oil, linseed oil or marinol K. However, in the same experiments serum Ig levels were significantly affected with samples from fish fed fosol and sunflower oil being significantly higher than samples from fish fed linseed oil and marine oil (Bell *et al.*, 1996). Therefore, the

requirements for different lipid qualities can vary according to the species studied and the stage of development.

Lipid metabolism is important in the acclimatisation of fish cell membranes to changing environmental temperature (Bell *et al.*, 1986), and therefore a few studies have focused on the effect of dietary lipids and varying temperature on immune response.
Channel catfish (*Ictalurus punctatus*) were the subject of studies in which the influence of dietary lipid and temperature on bacterial activity of macrophages were investigated (Sheldon and Blazer, 1991). At both temperatures studied, enhanced intracellular killing of bacteria could be correlated with increasing levels of (n-3) series fatty acids particularly long-chain HUFAs in the diet. However, Fracalossi and Lovell (1994) showed that channel catfish fed with menhaden oil and linseed oil for 9 or 13 weeks at 28°C had a lower survival than fish fed other diets containing lower percentages of (n-3) PUFAs and higher percentages of (n-6) PUFAs. Bly *et al.* (1990) studied the differential effects of temperature and exogeneous fatty acids on mitogen-induced proliferation in channel catfish T and B lymphocytes. They demonstrated that oleic acid (18:1) enhanced T cell responses at high temperatures (27°C) and rescued suppressed T cell responses at low temperature (17°C) by increasing membrane fluidity.

Finally, a few studies have focused on the dietary impact of both lipids and vitamin E on the immune response of Atlantic salmon. Fish fed with different levels of vitamin E and different percentages of (n-3) PUFAs for 5 months did not show any significant differences in their growth and mortality but the liver fatty acid composition and total lipid content was affected by the diets (Waagbø *et al.*, 1991). Another study this time using three different sources of oil (soybean, capelin or sardine oil) and two different levels of vitamin E (57 and 272 mg α-tocopherol/kg of diet) showed that after 10 months, salmon fed the highest levels of (n-3) PUFAs and vitamin E had significantly lower antibody levels following vaccination against *Vibrio salmonicida*. Total serum protein concentrations were not significantly affected, while total serum antibodies were somewhat higher in vitamin E supplemented groups. The coagulation activity was decreased in animals fed with increasing dietary (n-3) and a high vitamin E level (Waagbø

et al., 1993a). Salmon fed for nearly 12 months with these different oil sources showed reduced bacterial killing activity by isolated head kidney macrophages from fish fed high (n-3) PUFA (Waagbø *et al.*, 1993b). Another study focused on the effect of different vitamin C levels in combination with various (n-3) fatty acid levels and showed that juvenile salmon fed for 52 and 72 days with increased levels of (n-3) fatty acids had lowered antibody levels and likelihood of survival but an increased erythrocyte cell membrane strength (Erdal *et al.*, 1991).

Despite the abundance of data on the influence of lipid nutrition on growth and fatty acid composition of different tissues of turbot, only one investigation on the immunomodulation by dietary PUFAs and vitamin E has been carried out (Obach, 1993). Turbot of average weight 55 grams were fed for 34 weeks with 3 different levels of vitamin E and either cod liver oil or ground nut oil. At the end of the 34 weeks the growth, haematologic and plasma parameters and phagocytic activity did not show any significant difference between the dietary treatments. However, when progressively reducing the temperature to 10°C and prolonging the experiment for a further 12 weeks, differences in growth, number of leucocytes, lysozyme activity, chemiluminescence and lymphoproliferation of anterior kidney leucocytes were all affected by the dietary treatments, with a decrease generally observed for fish fed with groundnut oil compared to cod liver oil. Vitamin E does not seem to play a role in the variations observed except for the cell proliferation stimulated with PHA where higher levels of vitamin E increased the proliferation of anterior kidney leucocytes.

1.4 - AIMS OF THE STUDY

The aims of this study were to determine the optimal dietary vitamin E level required for an optimum immune response and to examine how vitamin E supplementation or depletion could affect some immune parameters in juvenile turbot (*Scophthalmus maximus*). Mostly non-specific parameters were studied and fundamental investigations were carried out on the haemopoetic organs to give an insight into the mechanisms of immunomodulation in this species of marine flatfish.

Vitamin E intake was also studied in relation to dietary lipids, firstly the oxidative state of lipids and secondly the nature of polyunsaturated fatty acids (PUFAs) as such components are known to be potential immunomodulators. An attempt to investigate which parameters are affected in each of these trials and in which way they are affected should give some more information on the role of dietary vitamin E and lipids in immunomodulation of turbot.

CHAPTER 2 - MATERIALS AND METHODS

2.1 - EXPERIMENTAL DESIGN

2.1.1- Experimental fish

Adult and juvenile turbot (*Scophthalmus maximus*) caught in Plymouth Sound were used for development of the assays.

The fish used in the feeding experiments, juvenile turbot, were obtained from Mannin Sea-Farm Ltd, Isle of Man. On arrival at Plymouth Marine Laboratory 16 fish on average were placed in each tank and allowed to acclimatise in the experimental system for two weeks prior to commencement of the experiment. During this period they were fed to satiation on a dry pellet similar in composition to the one used later for the experiment, except the level of α-tocopherol which was 80 mg/kg of diet.

On day (-1) of the experiment the fish were weighed and graded in the tanks to provide similar biomasses in each tank.

2.1.2 - Experimental system

2.1.2.1 - Water circulation system

2.1.2.1.1 - Experiment 1 and 2

The experimental system comprised eight 80 x litre circular poly-ethylene tanks each supplied with re-circulated sea-water (20°C ± 1.44, salinity 33‰ to 35‰) from the recirculating laboratory system at a flow rate of 100 l/hr. Water aeration was achieved by means of a double air stone in each tank. The exit water was drawing detritus from the bottom of the tank up through a stand pipe which maintained a fixed water depth.

2.1.2.1.2 - Experiment 3

Due to problems in regulation of water quality in the second experiment a new closed circuit was installed before starting the third experiment, with a biological filter (Project, external filter, new Project 2001) and a cooling system. The system was set up two months before receiving the fish and levels of nitrates and ammonia checked every day for the

appearance of the nitrite peak, an indicator of the build up of nitrifying bacteria inside the filter. Once the first peak was established individuals from Plymouth Sound were introduced into the closed circulation to produce waste and therefore induce the appearance of the second nitrite peak. Nitrification is sequential and nitrite oxidation does not proceed until ammonia conversion is well established. The result is a peak of ammonia and nitrite concentrations as the populations and activities of the different nitrifying species reach equilibrium with their energy sources (Spotte, 1992).

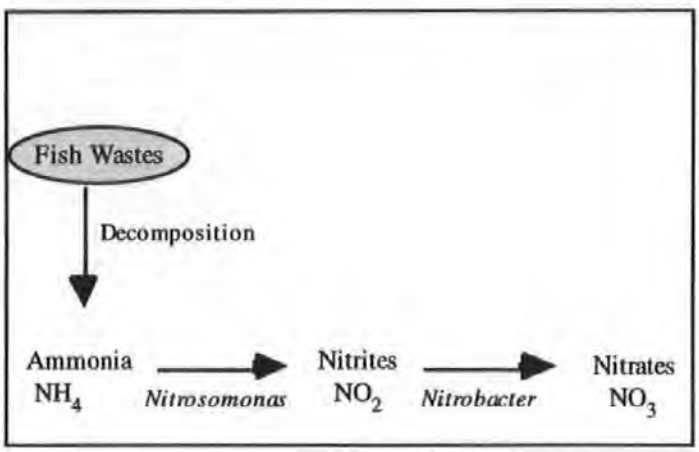


Fig. 2.1: Transformation of fish waste into nitrates

Once this was established, regular water changes were made to eliminate the nitrates which accumulated in the closed circulation.

The experimental system comprised eight 80 x litre circular white tanks but this time installed as a closed circulation with the biological filter and a cooling system to regulate the temperature. All tanks were supplied with sea-water (18°C ± 1, salinity 33‰ to 35‰) at a flow rate of 100 l/hr and individually aerated with oxygen.

2.1.2.2 - Water quality assessments

Levels of nitrites and nitrates were measured daily using Tetratest kits (Tetra Werke, Germany). The temperature was regulated with a cooling system but still measured regularly to investigate the level of variation.

2.1.3 - Experimental diets

2.1.3.1 - Basic composition of the diet

A generalised formulation is presented in Table 2.1.

Protein was supplied in the form of brown Chilean fishmeal (Crediton Feedmills, Crediton, Devon, U.K), meat and bone meal and blood meal, (Carne and Sons, Callington, Cornwall, U.K.).

The lipid contribution was partially supplied by the fish meal, though the bulk of the lipid was provided through cod liver oil ("Boost", Seven Seas Ltd., Hull, U.K.) or corn oil ("Mazola", C.P.C. Ltd., Esher, Surrey, U.K.).

Corn starch (Sigma, S-4126) constituted the carbohydrate component of the formulation.

Minerals were added to the diets in the form of a pre-mix, designed on the basis of maximum requirements of fish as described in N.R.C. and are shown in Table 2.3.

Pre-mixes were also used to provide the necessary vitamins in the diets. The fat soluble vitamins (except vitamin E), macrovitamins and watersoluble vitamins are shown in Table 2.2, 2.4, and 2.5. The vitamins were supplied by Roche Products Ltd. (Colborn Dawes, Heanor, Derbyshire, U.K.), Lonza U.K., Ltd. (Cheltenham, Gloucestershire, U.K.) and Takeda Chemical Company (Tokyo, Japan). All the premixes were prepared well in advance and stored at -20°C except the macro-vitamin premix which was prepared on the day of diet manufacture.

Vitamin E was supplied to the diet as a spray-dried acetate ester of α-tocopherol, available commercially as Rovimix E-50 SD (50% purity), Roche Products Ltd. (Colborn Dawes, Heanor, Derbyshire, U.K.). Actual inclusion levels of this vitamin varied between each investigation and are presented in each chapter.

Ingredient	Weight (g/kg of diet)
Fish meal	600
Meat and bone	100
Blood meal	50
Oil	100
Fat soluble vitamins	5
Water soluble vitamins	35
Mineral premix	15
Macrovitamins	10
Binder	5
Vitamin E + Filler	10
Corn starch	70

Table 2.1: Composition of the experimental diet (mg/kg).

Ingredient	Inclusion (mg/kg of diet)
Vitamin A	10
Vitamin D	4.8
Vitamin K	41.7
Wheat middlings	4943

Table 2.2: Fat-soluble vitamin premix composition (except vitamin E)

Mineral Salt		Inclusion (g/kg dry diet)
Calcium orthophosphate	$CaHPO_4.2H_2O$	12.000
Magnesium sulphate	$MgSO_4.7H_2O$	4.8450
Sodium chloride	NaCl	2.2800
Potasium chloride	KCl	1.9000
Iron sulphate	$FeSO_3.7H_2O$	0.9500
Zinc sulphate	$ZnSO_4.7H_2O$	0.2090
Manganese sulphate	$MnSO_4.4H_2O$	0.0960
Copper sulphate	$CuSO_4.5H_2O$	0.0298
Cobalt sulphate	$CoSO_4.7H_2O$	0.0181
Calcium iodate	$CaIO_3.6H_2O$	0.0112
Chromic chloride	$CrCl_3.6H_2O$	0.0048
Sodium selenite	Na_2SeO_3	0.0025
(α-cellulose)	(filler)	(27.6536)

Table 2.3: Mineral premix composition

Ingredient	Inclusion (g/kg of diet)
Choline Chloride	4
Inositol	4
Ascorbic acid	0.3
Corn Starch	1.7

Table 2.4: Macrovitamins premix composition.

Ingredient	Inclusion (mg/kg of diet)
Thiamine (B1)	12
Riboflavine (B2)	37.5
Pyridoxine (B6)	15
Pantothenic acid	50
Niacin	150
Biotin	4
Folic acid	12.5
Cyanocobalamin	20
Corn Starch	34504

Table 2.5: Water soluble vitamins premix composition

2.1.3.2 - Preparation of the food

Four experimental diets were designed and manufactured for each experiment as batches of 3 kg of food.

The dry powered ingredients (table 2.1) were individually weighed into a suitably sized container, then thoroughly mixed in the bowl of a Hobart bench food mixer, (model No. A120). The oil was then added very gradually in a continuous slow-pouring action, and finally tap water was added during continuous mixing to yield a dough considered sufficiently moist for extrusion (generally 300 cm^3 of water per kg of dry matter was used). Pellet extrusion was achieved using the extruder assembly of the Hobart processor, equipped with a 2 mm die. The resulting pellets were spread thinly on trays and air-dried at 35°C for 48 hours in a fan assisted drying-cabinet. The dried diets were then stored in plastic air-tight boxes and some samples were taken and stored at -70°C in air-tight plastic bags prior to analysis of proximate composition, α-tocopherol content, and in experiments 2 and 3, for fatty acid determination.

2.1.3.3 - Proximate chemical composition of the diets.

2.1.3.3.1 - Determination of moisture content.

Diets were ground into a powder using a laboratory grinder. About 5 grams of specimen were transferred into a clean foil tray previously labelled and weighed (WT) and the combined weight of sample and tray was recorded (WTS). The samples were then placed in a fan-assisted Pickerstone E 70F oven (R.E. Pickerstone Ltd., Thetford, Norfolk, U.K.) at 105°C for 24 hours. The samples were removed from the oven and allowed to cool in a dessicator before being finally weighed (WTF). The moisture content was calculated as follows:

$$\text{Moisture} = \frac{\text{Weight of the moist sample} - \text{Weight of the dry sample}}{\text{Weight of the moist sample}} \times 100$$

Where Weight of the moist sample = WTS - WT, Weight of the dry sample = WTF - WT

2.1.3.3.2 - Determination of the ash content

The diets were ground as described previously. The weights of crucibles were recorded (WC) and approximately 0.5 gram of sample to be tested was placed in each crucible and the total weight of sample + crucible recorded (WCS). The crucibles with specimen were then placed onto a tray and transferred to a Carbolite GLM 11/7 muffle furnace (Carbolite Furnaces Ltd., Bamford, Sheffield, U.K.) at 525°C for 12 hours for complete combustion of the sample and removal of all organic matter. The residue in the crucible was the inorganic non-combustible material or ash. Samples were carefully removed and placed in a dessicator to cool. The combined weights of crucible and ashes (WCA) were recorded and the percentage of ashes was calculated as follows:

$$\text{Ash} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

Where weight of the ash = WCA - WC and weight of sample = WCS - WC

2.1.3.3.3 - Determination of the crude protein by the Kjeldahl procedure

Five hundred mg of finely ground diet sample was accurately weighed and placed in a 250 cm^3 borosilicate-glass digestion tube. Two Kjeldahl catalyst tablets [2 x (3 g K_2SO_4, 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2)] (Thompson and Capper Ltd., Runcorn, Cheshire, U.K.) were added to the tubes to catalyse the digestion with 20 ml of concentrated H_2SO_4. Two supplementary tubes without samples are processed to act as blanks. The tubes were then carefully placed in a preheated Kjeldatherm digestion block (C. Gerhardt Laboratory instruments, Bonn, Germany) for 30 min at 250°C to completely dehydrate the samples. After the 30 min the temperature of the heating block was increased to 380°C to initiate the digestion and maintained for a further 90 min to complete digestion. The acid fumes were collected and neutralised by 15% NaOH in a Gerhardt Turbosog unit. The tubes were then left to cool down for 15 min during which time the digest turned to a grey colour. Then a Gerhardt vapodest 3S unit was used to carry out the distillation phase. Each sample was diluted with distilled water, neutralised by addition of NaOH (40%) and steam distilled to liberate the ammonia into the condenser. The liberated ammonia was then collected in a 250 cm^3 Ehrlenmeyer flask containing 50 cm^3 of saturated orthoboric acid (H_3BO_3) and 200 µl of BDH 4.5 indicator, resulting in a colour change from pink to blue. The ammonium borate solution was then titrated against 0.2 M HCl until the the receiving solution turned from blue to pink, at which point the amount of acid dispensed was accuately recorded. The crude protein content of the specimen was then calculated with the following equation:

$$\text{Crude protein} = \frac{[\text{sample titre} - \text{blank}] \times [\text{acid}] \times 14.0067 \times 6.25}{\text{sample weight}} \times 100$$

Where *sample titre* is the volume of acid in ml used to titrate the specimen, *blank* is the volume of acid in ml used to titrate the blanks, *14.0067* is the molecular weight of nitrogen, and *6.25* the Kjeldahl correction factor relating the amount of total nitrogen represented by protein.

2.1.3.3.4 - Determination of crude fat by Soxhlet extraction

Diet samples were homogenised using a laboratory grinder and dried in an oven at 105°C overnight. Approximately 5 grams of sample were placed in cellulose extraction thimbles (Whatman) previously weighed and a plug of glass wool placed in the neck of the thimble. The thimbles containing the samples were then placed into labelled and acurately weighed beakers containing anti-bumping granules. 130 ml of petroleum spirit (BDH, 40-60 fraction) was added to each beaker to dissolve the lipid in the samples. The beakers were then loaded into a Gerhardt Soxtherm-automatic machine and placed in contact with an aluminium hot plate at 150°C in the recirculation mode for 40 min during which time the lipids were dissolved and refluxed in the petroleum spirit. After the 40 min the machine was set to the mode recovery until the solvent had dropped to approximately 5 mm below the level of the extraction thimbles. Then the machine was set to the circulation mode for a further 70 min and to the recovery phase again until the solvent level containing the extracted lipids reached approximately 2-3 mm from the bottom of the extracting beaker. The beakers were then carefully removed from the machine and left under the fume hood overnight for complete evaporation of the solvent. Once all remaining solvent was completely evaporated the beakers were placed in an oven at 105°C for 30 min and left to cool in a dessicator for 15 min and subsequently weighed. The operation was repeated until the weight of the beakers varied by less than 0.1% of the original sample weight. The increase in weight of the beakers corresponds to the lipids of the samples and permits calculation of the proportion of lipids in all the samples as follows:

$$\text{Lipids} = \frac{W_f - W_i}{\text{Weight of the sample}} \times 100$$

Where W_f is the final weight of the beaker and W_i is the initial weight of the beaker. This allowed the calculation of lipid percentage per dry sample which further required adjustment to evaluate the amount of lipids per wet weight. This was done by applying the following formula:

$$\text{Lipids (\% wet weight)} = \text{Lipids (\% dry weight)} \times [1 - \frac{\text{moisture}(\%)}{100}]$$

Where *moisture (%)* is calculated with the formula from paragraph 2.1.3.3.1

2.1.3.4 - Determination of micronutrients.

2.1.3.4.1 - Determination of α-tocopherol by High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) was used to check the vitamin E levels in the diet, and in the liver which is the major stocking organ of vitamin E. This was carried out to ensure that the vitamin E included in the mixture during the preparation of the diets was present in the pellets and then that the vitamin E given to the fish through the diet was efficiently absorbed thus permitting a link to be made between the difference in the various parameters measured with the difference in vitamin E levels in the diets.

- *Diets*

Vitamin E content of the diets were determined by HPLC by the analytic service department of Roche (Basel, Switzerland) according to the method of of Manz and Philipp (1988).

- *Livers*

The livers were stored in sealed polythene bags at -70° C until use. After defrosting 1 g of tissue was weighed and homogenised with 9 ml of potassium chloride solution (1.15% KCl in distilled water) in a glass homogeniser. One ml of this homogenate was transferred into capped tubes and added to 2 ml of pyrogaloll (Sigma, P-0381) to prevent vitamin E oxidation. 300 µl of saturated KOH solution was added and the solution was mixed thoroughly before being placed in a 70°C water bath for 30 min to saponify. The tubes were placed on ice to cool and 4 ml of hexane (BDH, Analar, n-Hexane, 103876Q) supplemented with butylated hydroxytoluene (BHT, 0.005%) were added and thoroughly mixed to trap the vitamin E in the hexane phase. The tubes were then centrifuged at 1500 g for 10 min and the supernatant placed into other cooled Pyrex tubes on ice. The extraction operation was repeated a second time by adding 2 ml of hexane supplemented with BHT, centrifuging the mixture and transferring the supernatant to the cooled tubes again.

The tubes were placed in a 37°C water bath and perfused with nitrogen to avoid air contact and therefore vitamin E oxidation, until evaporation of hexane was completed. Then 500 µl of absolute ethanol (Sigma, 27-074-1) was added to the tubes to resolubilize the vitamin E. These solutions were then filtered through 0.2 µm nylon filter membranes (Whatman, cat No 7402001) into glass vials with a cap. The vials containing the samples were left in the freezer at -20°C until analysis.

A standard solution of α-tocopherol (Sigma, T-3251) at 20 µg/ml was prepared in ethanol (Sigma, 27-074-1). The standard and the samples were run through a Kontron HPLC system, equipped with a syringe-loading injector, a Hypersil BDS C18 column and a fluorescence detector (excitation 293 nm, emission 326 nm, sensitivity 500 V). Methanol (98%) was used as a mobile phase at a flow rate of 2.0 ml/min.

The calculations were carried out by peak area:

$$[\alpha - \text{Tocopherol}] = \frac{[\alpha - \text{T}] \times \text{Sample Peak Area} \times 500}{\text{Standard Peak Area} \times 1000}$$

The liver concentration of α-tocopherol (µg/g of tissue) can be calculated by multiplying the above value by 10 (total homogenate), then dividing by the recorded weight of tissue in the homogenate.

For experiment 3 vitamin E contents were determined HPLC by the analytic service department of Roche (Basel, Switzerland) following the protocol described by Hesse *et al.*, (1991).

2.1.3.4.2 - Determination of fatty acids by gas chromatography (GC)

- *Diets*

After manufacturing, samples of diets were taken and kept in the freezer at -70°C until analysis with gas chromatography for fatty acid composition determination.

Three to 5 pellets were taken from each diet and placed in glass tubes containing 4 ml of a 2:1 chloroform (BDH, AnalaR, 10077 6B) : methanol (BDH, AnalaR, 10158 6B) solution. The tubes were then sonicated for 10 min to facilitate lipid extraction. The solution obtained was filtered using Whatman filter paper (90 mm Ø, Cat. No. 1001090) into glass

Teflon sealed cap vials. A solution of 0.88% KCl W/V (BDH, AnalaR, 10198) was added in a ratio 1/4 mixed thoroughly and centrifuged for 5 min at 200 g. The upper layer containing water was then discarded, the chloroform layer containing lipids evaporated under nitrogen and the tubes placed in a dessicator for 30 min. One ml of toluene and 2 ml of a solution of 1% sulphuric acid in methanol were added to the tubes which were subsequently shaken thoroughly. The tubes were placed in a heating block at 50°C and left to methylate for 15 hours.

At the end of the methylation period, the tubes were removed from the heating block, allowed to cool and the solution transferred to cap test tubes. Four ml of distilled water + 4 ml of a (1:1) solution of hexane (BDH, AnalaR, 10387 6Q) : diethyl ether (BDH, AnalaR, 10094 6B) were added and shaken thoroughly before centrifuging at 200 g for 5 min. The upper layer containing lipids was pipetted up and transferred into cleaned glass tubes; 2 ml of $KHCO_3$ solution (Sigma, P-4913) (2%W/V) was added, the tubes shaken thoroughly and centrifuged for 5 min at 200 g. The upper layer containing lipids was pipetted up and moved to Teflon sealed vials where the solution was blown down using nitrogen. The lipids were then resuspended in a small volume of hexane + butylated hydroxytoluene.

Thin layer chromatography (TLC) was used to separate the polar lipids from neutral lipids and extract the fatty acid methyl ester of interest. The solutions were run on TLC glass plates (20 x 20 cm) coated with silica gel using 90 ml of hexane + 10 ml of diethyl ether and 1 ml of acetic acid as a developing solvent. Plates were left in the dessicator for 5 min to dry, developed chromatograms were sprayed with a saturated solution of dichlorofluorescein (Sigma, D-6665) in methanol, and the lipids visualised under UV light. The bands of adsorbent containing methylated fatty acids, saturated and unsaturated, were scraped off the plates into glass test tubes containing 2 ml of $KHCO_3$ solution (2%W/V) and 4 ml of a (1:1) solution of hexane:diethyl ether. The mixture was centrifuged at 200 g for 5 min and the upper layer containing the methylated lipids pipetted up and transferred into cleaned Teflon sealed vials. The solution was evaporated with nitrogen and lipids were resuspended into 500 µl of hexane. This solution was then transferred into 2 ml glass vials previously weighed and evaporated again with nitrogen. The vials were then weighed and the amount of lipids calculated and resuspended in an adequate volume of hexane to give a concentration of 2 mg of lipids per ml of solution.

The samples were left in the freezer at -18°C before being run through a Carlo Erba strumentazione, HRGC 5160 gas chromatograph equipped with a Restek fused silica capillary column, 30 m of length and 0.32 mm of internal diameter, coated with Stabilawax phase 0.25 μm film thickness. Hydrogen was used as a carrier gas and samples were injected via cold on column injection. The oven temperature was programmed to rise from 50°C to 190°C at a rate of 40°C per min then from 190°C to 220°C at 1.5°C per min. The oven temperature was then held at 220°C for a further 15 min. Individual fatty acid methyl esters were detected by a flame ionisation detector and the peaks identified by reference to a well characterised marinol standard. The results were calculated as follows:

$$\% \text{ Composition} = \frac{\text{FA area}}{\sum \text{FA areas}} \times 100$$

Where *FA area* is the calculated area for a fatty acid peak in the chromatograph and $\sum$ *FA areas* is the sum of fatty acid areas identified on the chromatograph.

- *Livers*

The livers were dissected out, placed into plastic sealed bags and kept in the freezer at -70°C prior to lipid extraction. After being defrosted the livers were then homogenised using a Janke & Kunkel homogeniser (Ultra Turrax Thyristor) in 6 ml of a (2:1) solution of chloroform:methanol. The following extraction steps were similar to the ones described in the diet protocol above.

2.2 - GROWTH PARAMETERS

2.2.1 - Growth measurement

The fish were fed each test diet twice daily to satiation. The biomasses and feed intakes were recorded once every two weeks. To avoid stress fish were weighed in groups of four or five in order to reduce the time of manipulation. The average weight of fish per

treatment was calculated and a graph was established where the average weight was plotted against time throughout the experimental feeding.

2.2.2 - Specific growth rate (SGR)

Specific growth rate is used to compare the growth of fish on a relative daily basis. It is expressed as the mean percentage increase in body weight per day over a given time period. In this study the specific growth rates were calculated for a two week period corresponding to the times at which the fish and food distributed were weighed.
The specific growth rates were calculated as follows:

$$SGR = \frac{LogeW_2 - LogeW_1}{T_2 - T_1} \times 100$$

where W_2 is the fish mean weight in grams at the time T_2, W_1 the fish mean weight in grams at the time T_1, T_2-T_1 was measured in days (Ricker, 1979).

2.2.3 - Feed Conversion Ratio (FCR)

Feed efficiency may be expressed as Feed Conversion Ratio. This term is widely accepted in practical fish nutrition trials. The food conversion ratio gives the mean ratio of food fed converted into live weight.
It was calculated every two weeks as follows:

$$FCR = \frac{\text{Dry Food Fed}}{\text{Live Weight Gain}}$$

where *dry food fed* is the weight of food fed to the fish in grams and *live weight gain* represents the weight gained by the fish in grams over the same period.

2.3 - ASSAYS FOR IMMUNOCOMPETENCE

2.3.1 - Cellular

2.3.1.1 - Blood collection

Blood samples were taken from the caudal vein of unanaesthetised fish held in a damp towel, using non-heparinised sterile 1ml syringes fitted with 0.5 x 25 mm needles. Samples were immediately used to make blood smears or transferred to plastic Eppendorf tubes prior to serum separation. Following overnight storage at 4°C and centrifugation at 1000 g for 10 min, serum samples were decanted and stored at -70°C prior to protein and lysozyme analysis.

2.3.1.2 - Leucocrit and haematocrit

Immediately after being sampled, blood was collected into heparinised tubes (Hawksley, BDH), closed with cristaseal (Hawksley, cat. No. 01503) and the tubes were centrifuged for 15 min at 14000 g in an haematocrit centrifuge (Jouan, Hema-C). The tubes were taken out of the centrifuge and immediately read on the scale to determine the percentage of red blood cells (haematocrit) and white blood cells (leucocrit).

2.3.1.3 - Differential leucocyte counts

Blood smears were prepared on methanol-cleaned slides, air dried and fixed in methanol before being stained with May-Grunwald (BDH, Cat.No. 35025-5S) and Giemsa (BDH, Cat. No. 35014-4M) stain. Following staining, slides were viewed under oil immersion at x100 and the number of lymphocytes, thrombocytes and phagocytes (including monocytes and neutrophils) recorded per 200 leucocytes and expressed as a percentage of total leucocytes.

2.3.1.4 - Cell separation

Following dissection of the kidney and spleen the organs were pushed through a 40 μm wire mesh, using a plastic rod, into a petri dish containing 2 ml of Leibovitz L-15 culture medium (Sigma, L-5520) supplemented with 4% foetal bovine serum (Jansen, Belgium), 1000 μg/ml streptomycin, 100 U/ml of penicillin (GibcoBRL, Cat. No. 15140-114) and 40 IU/ml heparin (Sigma, H-3193). The cell suspensions were placed very carefully on the surface of 6 ml of lymphocyte separation medium (Flow laboratories) in 12 ml plastic tubes and centrifuged at 200 g for 30 min in a cooled bench top centrifuge (Jouan, CR3-12)

The layer containing the white blood cells was collected with pipettes and placed in 5 ml plastic tubes with flounder ringer (Wasserman *et al.*, 1953) and centrifuged for 10 min at 60 g to remove excess separation medium. The supernatant was then pipetted off and the cells resuspended in 1 ml of L-15 supplemented medium, phosphate buffered saline (Gibco BRL, Cat. No.14200-067) or RPMI without phenol red (Sigma, R-7509) according to the assay.

2.3.1.5 - Phagocytosis assay

For the phagocytosis assay an adaptation of the procedure of Pipe *et al.* (1995) was used. The cells obtained according to the cell separation protocol were plated in duplicate wells at 50 μl per well and left in the constant temperature room 10°C for one hour to adhere using flat bottomed microplates (Greiner Ltd). Some wells were plated in duplicate for each treatment to act as negative controls and another duplicate to act as the blanks. The supernatant was removed by inverting the plate upon some absorbent paper and the cells were rinsed twice with PBS pH 7.2. For the incubation with the dyed zymosan a time exposure of 30 min was chosen according to the previous studies. The cells were incubated with 50 μl/well of zymosan solution (5 x 10^8 particles/ml) (Sigma, Z-4250) stained with neutral red (Sigma, N-2880). Ten min before the end of attachment period 100 μl of Bakers formol calcium was placed in the wells designed for the negative controls to fix the cells. After 30 min of incubation with dyed zymosan the reaction was stopped by adding

100 µl of Bakers formol to all the wells for 10 min. The plate was then spun down at 60 g for 5 min, the supernatant pipetted off carefully and the cells washed several times in PBS until the negative controls were clear. Before the last centrifugation a range of concentrations of dyed zymosan (50, 25, 12.5, 6.25, 3.125 and 1.5625 $x10^8$ zymosan particles/ml) were added in duplicates to the plate to produce a standard curve. The dye was then resolubilised using 100 µl/well of acidified ethanol (50% distilled water with 1% acetic acid + 50% ethanol) for 10 min and the plate read at 550 nm. A model was established from the standards as follows:

$$[OD] = b_0 + b_1 [n_0 \text{ of zymosan particles}]$$

where OD is the optical density measured, b_0 the intercept and b_1 the slope. The replacement of OD in this equation by the optical densities measured allowed an estimation of the number of zymosan particles ingested per well.

At the same time a protein plate (section 2.316) was processed following the conditions of the assay to determine the protein concentration in each sample. Finally by dividing the result obtained above by the protein concentration, the phagocytosis results were calculated, and expressed as the number of zymosan particles ingested /mg of protein.

2.3.1.6 - Protein assay for the phagocytosis assay

2.3.1.6.1 - Expression of cell number

This assay is performed to give an evaluation of the number of cells used in the functional assays, such as the phagocytosis assay or pinocytosis assay, using the microplate reader. This allows the assay to proceed without having to count the different cell suspensions, and therefore avoids a long waiting time which could disrupt cell physiological processes. However, in order to be able to correlate the number of cells with the protein concentration it was necessary to test the relationship between known cell concentrations in the wells with protein concentration readings in those wells.

A microplate was processed for protein determination with duplicate wells containing cells taken from spleen and kidney of turbot and diluted in a range of increasing concentrations.

The plate was then processed for protein determination as indicated in the following paragraph.

The data showed good correlation between the protein concentration (Fig. 2.2) allowing expression of the result as O.D values per mg of protein. The correlation coefficient ($r = 0.956$) was significant for $p < 0.01$

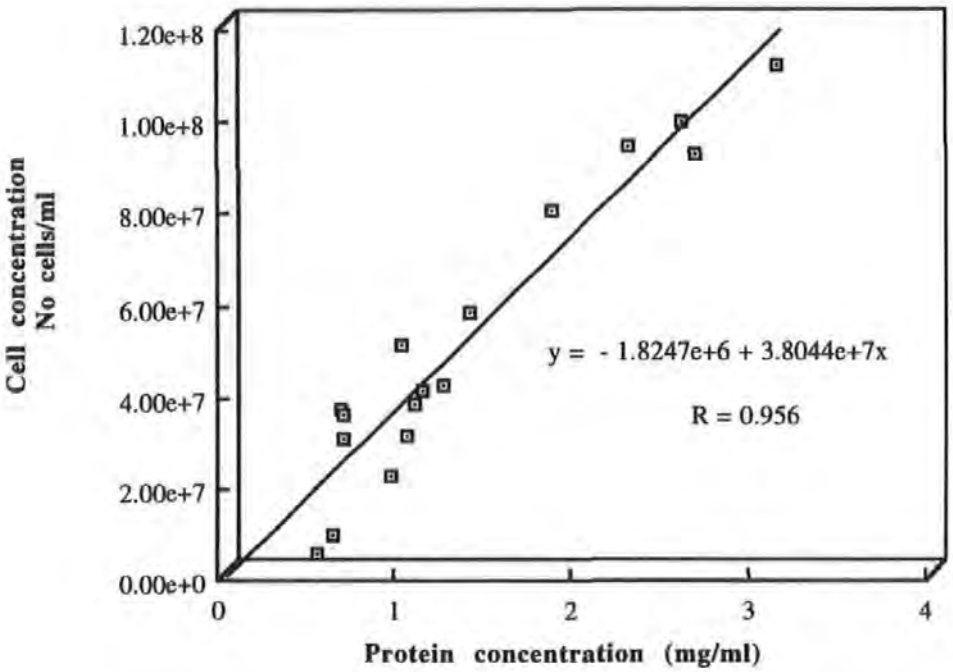


Fig.2.2: Correlation between the protein concentration and cell concentration

2.3.1.6.2 - Protein assay

The cells were obtained using the same preparation as the one used for the phagocytosis assay. They were plated in a microplate at 50 µl per well in duplicate for each tissue sample and left for one hour to attach at 10°C. The supernatant was then tipped out, and the wells rinsed twice using PBS. Fifty µl of PBS added to all the wells + 50 µl of CHAPS (3[(3-cholamidopropyl)-dimethylamonio]1-propanesulphonate) (Pierce, Cat. No. 28300), and the cells resuspended by pipetting up and down thoroughly; the plate was then incubated at 37°C for 30 min.

Following the incubation 10 µl of each well was pipetted off the plate and placed in duplicate in a new microplate. Four wells were filled with 10 µl of distilled water as blanks and another aliquot of wells in duplicate were filled with 10 µl of the prepared set of

protein standards (100, 200, 400, 600, 800 and 1000 µg/ml of bovine serum albumin in distilled water). The standards were prepared by diluting serum albumin protein in different quantities of distilled water and placed as 1 ml aliquots in Eppendorf tubes and stored at -20°C until required for the assays. Two hundred µl of working reagent (obtained by mixing 1 part of reagent A into 50 parts of reagent B (BCA protein assay reagent, Pierce Cat. No. 23225) was added to all the wells and the plate was incubated for a further 30 min at 37°C. The plate was read immediately at 562 nm wavelength in the microplate reader, a standard curve was established and a regression model calculated as follows:

$$[OD] = b_0 + b_1 [Prot.Conc.]$$

where *OD* is the Optical Density read, b_0 the intercept, b_1 the slope and *Prot.Conc.* the concentration of protein in µg/ml.

The replacement of OD in this equation by the optical densities measured for each sample allowed to estimate the protein concentration of each sample which is proportional to the number of cells attached to the plate.

2.3.1.7 - Uptake of neutral red assay

For the neutral red assay an adaptation of the procedure of Borenfreund and Puerner (1985) was used. The macrophages were separated according to the method described in section 2.3.1.4 and resuspended in 1 ml of flounder ringer before being plated in duplicate wells at 50 µl per well in duplicate and left for one hour at 10°C to attach. The supernatant was then removed by inverting the plate onto some absorbent paper and the wells rinsed twice using PBS. Fifty µl of PBS + 10 µl of a neutral red solution (0.33% neutral red (Sigma N-2880) in phosphate buffered saline, PBS pH: 7.2 (BDH Gurr, 33194 2F)) was added to all wells including a duplicate of wells for each treatment with 50 µl of PBS without cells + 10 µl of neutral red for the blanks. The plate was incubated for one hour at 10°C. After this time the plate was centrifuged at 150 g for 10 min, the supernatant removed and the cells fixed with 100 µl of Bakers formol calcium per well for 10 min. The Bakers formol calcium was removed after centrifuging the plate and the cells were washed

twice with PBS or Flounder Ringer and centrifuged in between to remove the neutral red not taken up by the cells. One hundred µl/well of acidified ethanol was added to resolubilize the dye before reading the microplate at 550 nm.

Another plate was set up under the same conditions at the same time to measure the protein concentration which was calculated as indicated in section 2.3162. The uptake of neutral red results were finally calculated by dividing the optical densities read by the corresponding amount of protein and expressed as OD values per mg of protein.

2.3.1.8 - Cell proliferation assay

The lymphocytes were separated according to the method described in section 2.3.1.4 and resuspended in 1 ml of RPMI (Sigma, R-7509). The cells were then plated at 50 µl per well with duplicates for each treatment. Fifty µl of test solutions with different stimulants (Concanavalin A (Sigma C-2010), 50 µg/ml, lipopolysaccharide (Sigma L-30120), 200 µg/ml, pokeweed (Sigma L-9379), 5µg/ml, were then added to each well containing the cells. For each test solution two wells were filled with 50 µl of culture medium and 50 µl of the test solutions for the blanks.

At the same time another microplate was set up with 50 µl of each cell sample + 50 µl of RPMI and two wells filled with 100 µl of RPMI for the blanks. This plate was used as the base line to calculate the number of cells in the culture at the beginning of the experiment. However to avoid variation of cell concentration between the different individuals to be tested cells suspensions were counted and the concentrations were adjusted to 2 x 10^6 cells/ml.

A solution of 100 µl PMS (phenazinemethiosulphate in Dulbecco's phosphate buffered saline, pH 7.3) and 2 ml of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salts (MTS) (2.0 mg/ml in Dulbecco's phosphate buffered saline, pH 6.0) was used as a dye solution to read the plate (non radioactive cell proliferation assay- Promega, Cat. No. G5421). The solution was added in 20 µl aliquots to every well including the blanks. The plate was incubated for two hours with the dye solution at 10°C before being read at 492 nm. The other plates with the stimulants were incubated for two days at 10°C before being incubated with the dye and read at 492 nm

with the microplate reader. The calculations were carried out to express the results as stimulation index (SI). First the number of cell divisions over a period of time was calculated using the following formula:

$$n = \frac{LogB - LogA}{Log2}$$

Where n is the number of divisions in the period of culture, B is the optical density read after culture and A is the optical density read before culture. A and B are proportional to the number of viable cells present in each sample well. As the cells divide according to a geometric progression the following equation is derived:

$$A \times 2^{(n)} = B$$

By taking the logs to the base 10 the formula described above is obtained.
Then the stimulation index was evaluated by dividing the number of divisions obtained when using a mitogen for stimulation of proliferation, by the number of divisions obtained without using any mitogen (control).

$$SI_{conA} = \frac{N_{ConA}}{N_{Control}}$$

where SI is the stimulation index and N is the number of divisions.

2.3.1.9 - NBT assay

The cell suspensions obtained as described in section 2.3.1.4 were plated at 100 µl/well in a microplate in as many replicates as necessary and allowed to attach for an hour at 10°C. After this the solutions were tipped off the plate and 100 µl of nitroblue tetrazolium (NBT) (Sigma, N-6876) solutions (2 mg/ml of PBS) were added to the wells. Three different solutions were added :

- without any stimulant for control

- with 50 µg/ml of Concanavalin A for stimulation
- with both stimulant, Con A (50 µg/ml), and inhibitor, superoxide dismutase (SOD) (1000 Units/ml) (Sigma, S-2515)

For each different treatment two wells of blanks were made without cells. The cells were incubated with these solutions at 4°C for 15 min. The solutions were then removed, and the plate rinsed twice with 100 µl of PBS per well to ensure that all the NBT not taken up by the cells was removed. The cells were then fixed with 100% methanol and the plate rinsed three times with 100 µl of 70% methanol in each well. The plate was allowed to air dry before resolubilising the blue formazan with 120 µl of 2M KOH and 140 µl of dimethyl sulphoxide (DMSO), (Sigma, D-5879). After ten min the microplate was read at 620 nm. The results were expressed as OD values/mg of protein.

2.3.1.10 - Protein assay for the NBT assay

The cells were obtained using the same preparation as the one used for the NBT assay. The cells were plated in a microplate at 100 µl per well in duplicate for each of the tissue samples and left for one hour to attach at 10°C. The supernatant was then tipped out, 50 µl of PBS added to all the wells + 50 µl of CHAPS (3[(3-cholamidopropyl)-dimethylamonio]1-propanesulphonate, Pierce), and the cells resuspended by pipetting up and down thoroughly; the plate was incubated at 37°C for 30 min. After the incubation, 10 µl of each well was pipetted off the plate and transferred in duplicates to a new microplate. Four wells were filled with 10 µl of distilled water as blanks and another aliquot of wells in duplicates were filled with 10 µl of the prepared set of protein standards (100/200/400/600/800 and 1000 µg/ml of bovine serum albumin in distilled water). Two hundred µl of working reagent (obtained by mixing 1 part of reagent A into 50 parts of reagent B from the BCA protein assay reagent, Pierce) was added to all the wells and the plate was incubated for a further 30 min at 37°C. The plate was read immediately at 562 nm in the microplate reader. The results were expressed as mg of protein /ml of solution.

2.3.1.11 - Ferricytochrome C assay

The macrophages were obtained as described in section 2.1.3.4 and resuspended in 1 ml of flounder ringer before being plated at 50 µl per well in duplicate by treatment. Three different treatments were made

- control with cells and no stimulant or inhibitor
- with 50 µg/ml of Con A as stimulant (Sigma C-2010)
- with both Con A (50 µl/ml) and SOD (1000 units/ml) (Sigma S-2515))

50 µl of each solution were added to the wells and 50 µl added to 50 µl of flounder ringer in duplicate for each treatment for the blanks. The microplate was read at 550 nm wavelength in a microplate reader for one hour every 10 min.

At the same time a plate was performed under the same conditions for the protein assay and the results were expressed as OD values/mg of protein and plotted against time.

2.3.2 - Humoral

2.3.2.1 - Lysozyme assay

Serum samples were taken out of the freezer and left at room temperature to defrost. Then 10 µl of each serum sample was plated in triplicate for each fish. Two hundred µl of 0.3 mg/ml *Micrococcus lysodeikticus* (Sigma M-3770) was added to all the wells except for the blanks where only 260 µl of sodium phosphate buffer (SPB) were placed in 4 wells. The plate was read 30 seconds after adding the bacterial solution at 450 nm wavelength and then each min for 10 min.

Another plate was set up with a prepared set of hen egg white lysozyme (HEWL) (Sigma L-6876) standards. The HEWL powder was diluted in SPB 0.07M, pH:6.2 (40.7 ml of NaH_2PO_4 2.75% solution + 9.3 ml of Na_2HPO_4 2.83% solution + 50ml of distilled water) to obtain a range of 0, 2, 4, 6, 8, 10 & 20 units/well. 10 µl of these solutions were plated in triplicate and 250 µl of the bacterial solution was added to all the wells except for the blanks where 260 µl of bacterial solution were added to only 4 wells. The plate was read 30 seconds after adding the bacterial solution and then each min for 10 min. The average

variation of optical density per min was calculated over this ten min period and the results were plotted as the ΔOD/min against the number of hen egg white lysozyme units/well. Parameters b_0 and b_1 (intercept and slope) of a linear regression model were calculated as follows:

$$[\text{Delta OD / min}] = b_0 + b_1[\text{HEWL Units / ml}]$$

The HEWL equivalent units/ml are then estimated for each sample as follows:

$$[\text{HEWL Units / ml}] = \frac{[\text{Delta OD / min}] - b_0}{b_1}$$

Then the average activity for each dietary treatment was calculated.

2.3.2.2 - Serum protein assay

Stored serum samples were defrosted and diluted 1:50 in distilled water before starting the assay in order to obtain a suitable concentration of protein for the reading. Ten µl duplicates of the diluted serum sample from each fish were placed in a microplate, 10 µl of distilled water was placed in four wells for the blanks and 10 µl of a prepared set of protein standards in duplicate in two columns of wells (100, 200, 400, 600, 800 & 1000 µg/ml of bovine serum albumin in distilled water (Pierce)). 200 µl of working reagent (1 part of reagent A with 50 parts of reagent B, BCA protein assay reagent, Pierce), was added to all the wells and the plate incubated in the oven at 37°C for 30 min. The plate was read immediately after the incubation at 562 nm and the concentration of protein calculated from the standard curve (see section 2.3.1.6.2) and finally expressed as mg of protein per ml of serum.

2.4 - FLOW CYTOMETRY

The separated cells remaining from the different assays were used for flow cytometry examination. The cells were obtained according to the protocol described in section 2.3.1.4 and the suspensions remaining after providing cells for all the assays and fixed using Bakers formol calcium. The samples were rinsed twice with flounder ringer by centrifuging at 300 g for 10 min and resuspended in flounder ringer the day of analysis. Some samples were kept for fluorescent labelling using FITC labelled Pokeweed or concanavalin A. For that purpose the cell suspensions were left overnight with the different lectins at a concentration of 5 µg/ml for pokeweed and 50 µg/ml for Concanavalin A. Just prior to examination the cell suspensions were rinsed twice by centrifuging at 185 g for 10 min.

The cell suspensions were then processed through the fluorescence activated cell sorter (FACS) (Becton Dickinson- PC-Lysis) and examined using different parameters: side scatter, forward scatter or fluorescence for the labelled cells.

2.5 - STATISTICAL ANALYSIS

Data are expressed as the mean of the individual tested ± standard errors. The distribution of the population were examined and where non-normal distribution was obtained for at least one of the group non-parametric test Kruskal-Wallis (Kruskal and Wallis, 1952) was applied for determination of significance. Data where considered significantly different for $p < 0.05$.

When statistical difference was characterised with Kruskal-Wallis test, pairwise comparison were carried out with Mann and Whitney non parametric test. In order to achieve the experimentwise error rate α' was calculated according to the the Dunn-Sidak method (Ury, 1976) as follows:

$$\alpha' = 1 - 1(1 - \alpha)^{1/k}$$

where $\alpha = 0.05$ and k is the number of comparisons.

CHAPTER 3 - EFFECT OF VITAMIN E ON THE IMMUNE SYSTEM OF TURBOT (*SCOPHTHALMUS MAXIMUS*)

3.1 - INTRODUCTION

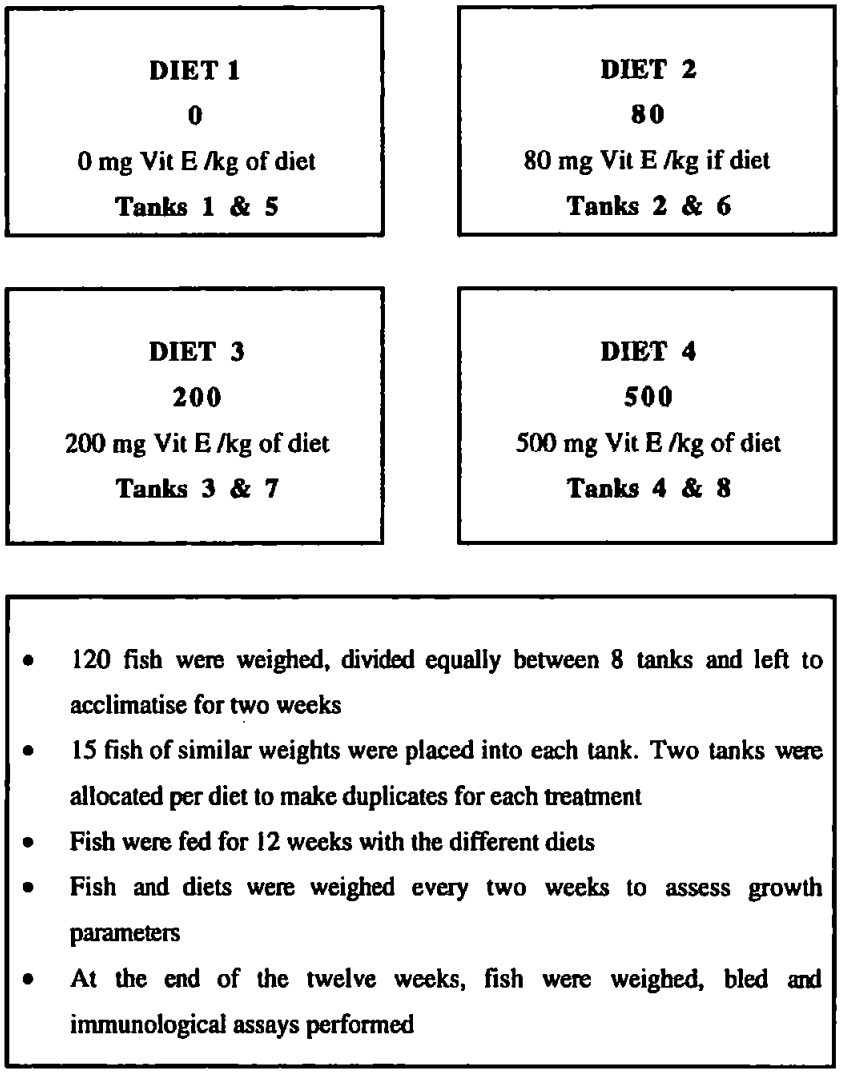
Vitamin E is a fat soluble vitamin found at a low level in cell membranes. Unlike other vitamins, it does not seem to have a specific enzyme function, but is the major, lipid soluble, free radical trapping antioxidant in membranes (Bender, 1992). The side chain of α-tocopherol can interact closely with some long chain polyunsaturated fatty acids. It thus stabilises membrane structure by preserving membrane impermeability and provides some protection against damage by enzymes such as phospholipase (Diplock and Lucy, 1973). Only very small amounts of α-tocopherols are required for these membrane specific actions. Vitamin E is one of the most active radical trapping and chain breaking antioxidants (Burton and Ingold, 1981, 1984). Alpha-tocopherol can be reduced to α-tocopheroxyl radical when trapping an oxyradical and later can be reduced by ascorbic acid (Vitamin C) thus regenerating its antioxidant functions (Bender, 1992). Dietary vitamin C and E supplements have been shown to be a necessary supplement to the diet to ensure immune competence and increase disease resistance in several commercially important fish (Blazer and Wolke, 1984; Hardie *et al.*, 1990; Blazer and Wolke, 1991; Hardie *et al.*, 1991; Verlhac *et al.*, 1991; Furones *et al.*, 1992; Wise *et al.*, 1993; Verlhac and Gabaudan, 1997). However, although most studies seem to agree that a deficiency of vitamin E in the diet is detrimental to the health of the animal, only a few point out a beneficial effect of higher dietary levels on immune parameters in fish.

Previous work has been carried out on turbot to study the combined effect of vitamin E and C on the immune system. Some cases of disease occured in fish fed both a vitamin E and C depleted diet, suggesting that supplementing the diet with both vitamins provided better resistance to infection.

The present study was undertaken to investigate the effects of different dietary levels of vitamin E on some immunological parameters of turbot in order to investigate the effect of vitamin E independently of vitamin C. This study should also enable a determination, not only of the specific requirement of juvenile turbot for vitamin E, but also of any relationship between the fed dose of vitamin E and selected immune parameters.

3.2 - EXPERIMENTAL PROTOCOL

The following figure summarises the experimental design.



3.3 - COMPOSITION OF THE EXPERIMENTAL DIETS

3.3.1 - Chemical composition of the diets

The ash, moisture, protein and lipid composition of the diets were determined by the methods described in paragraph 2.1.3.3. The results are presented in Table 3.1.

Diet	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
0	6.9	11.1	42.5	13.5
80	5.7	11.7	43.0	12.6
200	6.9	11.1	42.6	13.6
500	4.6	11.0	41.4	11.0

Table 3.1: Composition of practical test diets expressed as percentage of wet weight

On average the diets were composed of 11.2% ash, 6% moisture, 42.4% protein and 12.7% lipids. Although the same levels of minerals, lipids and proteins were added to all the diets variations were measured in the chemical composition between the different diets after manufacture.

3.3.2 - Analysis of vitamin E levels

Vitamin E levels in the diet were measured by HPLC (ROCHE, St Louis, France). The data are presented as the average of 2 replicates in Table 3.2.

Diet	Included (mg/kg)	Measured (mg/kg)
0	0	3
80	80	45
200	200	108
500	500	339

Table 3.2: Vitamin E levels of diets after manufacture

3.4 - UPTAKE OF VITAMIN E

The results of HPLC analysis of the vitamin E content in livers are presented in Table 3.3. N represents the number of fish sampled per treatment and the data are presented as the mean ± 1 SE

The results show a clear difference between fish from the four treatments (Kruskal-Wallis test, p = 0.0001) and the amount of vitamin E found in the livers showed a positive correlation with the amount of vitamin E given in the diet (r = 0.882, p < 0.01)

Diet	N	Dietary vitamin E (mg/kg)	Liver vitamin E (µg/kg)
0	11	3	11.8 ± 6.5^{a}
80	14	45	58.5 ± 8^{b}
200	11	108	178 ± 17^{c}
500	12	339	397 ± 44^{d}

Table 3.3: Levels of vitamin E in the livers of turbot (*S. maximus*) after 12 weeks of feeding with different vitamin E levels. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

3.5 - GROWTH PERFORMANCE

3.5.1 - Growth

The weight and length of each individual fish was recorded at the end of the experimental trial and the data are shown in Table 3.4. N represents the number of fish sampled per treatment and the data are presented as the mean ± 1 SE.

Diet	N	Weight (g)	Length (cm)
0	21	34.2 ± 2^{a}	13.2 ± 0.3^{a}
80	26	45.4 ± 2^{b}	14.3 ± 0.2^{b}
200	20	47.0 ± 2^{b}	14.3 ± 0.2^{b}
500	23	42.4 ± 3^{ab}	13.8 ± 0.3^{ab}

Table 3.4: Weight and length of turbot (*S.maximus*) at the end of the feeding trial. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

Statistical analysis using the Kruskal-Wallis test showed significant differences between the dietary treatments for both final fish weight (p = 0.0015) and final fish length (p = 0.014). Differences between individual groups are indicated by superscript letters in the table.

The growth performance of turbot during the twelve weeks of feeding are shown in Fig. 3.1. As fish were weighed in groups of 5 to avoid handling stress, standard errors could not be calculated.

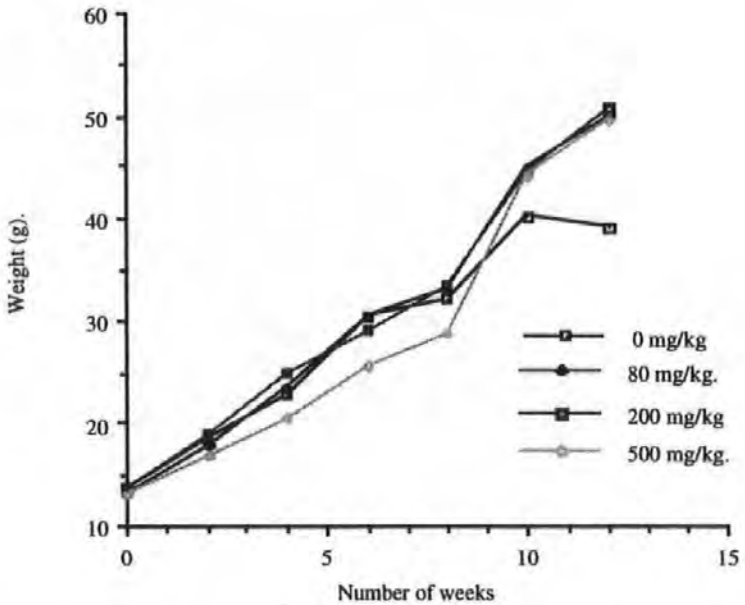


Fig. 3.1: Growth performance of turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E

3.5.2 - Specific Growth Rate (SGR)

The cumulative SGR data are presented in Table 3.5. The data are presented as the mean of the two replicates ± S.E. and N represents the total number of individuals (sum of the two replicates) for each treatment at the end of the experimental period.

Diet	N	SGR	FCR
0	21	1.0 ± 0.06	1.5 ± 0.2
80	26	1.6 ± 0.06	0.9 ± 0.005
200	20	1.2 ± 0.45	1.6 ± 0.7
500	23	1.4 ± 0.17	1.0 ± 0.1

Table 3.5: Specific growth rate and food conversion ratio of turbot (*S. maximus*) fed for 12 weeks with diets containing different levels of vitamin E

Statistical analysis did not detect any differences between dietary treatments (Kruskal-Wallis, $p < 0.05$).

3.5.3 - Food Conversion Ratio (FCR)

The cumulative FCR was calculated for the total length of feeding period as the mean of the two replicate tanks ± 1 S.E, and is presented in Table 3.5. Statistical analysis did not detect any differences between dietary treatments (Kruskal-Wallis, $p < 0.05$).

3.5.4 - Behavioural recordings

During the first two weeks of the experiment, fish were rapidly conditioned to come to the surface to obtain food when the lids of the tanks were opened. This became a habit and occurred as soon as the lids were opened, even in the absence of feeding. During the first weeks of feeding the fish appeared well settled and food consumption increased progressively.

However, after a few weeks, a difference in appetite was apparent between fish from different tanks. Turbot fed on a depleted or low vitamin E diet were eating more than fish fed on diets with higher vitamin E levels. This behaviour was not apparent at the end of the trial.

After five weeks of treatment, a marked change in colour from brown to black was noticed in some fish fed on the depleted diet that extended to all the individuals by the end of the trial. This change was followed by a problem in the feeding behaviour. Fish appeared to have problem with vision, failing to catch pellets and swimming into obstacles. Some of the fish fed on a depleted vitamin E diet (0 mg/kg) appeared to die as a result of malnutrition and showed signs of infection prior to death.

3.6 - ASSAYS FOR IMMUNOCOMPETENCE

3.6.1 - Cellular assays

3.6.1.1 - Haematocrit

Haematocrit, leucocrit and plasma percentages are presented in Table 3.6. N represents the number of fish sampled per treatment and the data are presented as the average ± SE.

Diet	N	Haematocrit	Leucocrit	Plasma
0	19	19.5 ± 0.7 ^{a}	0.9 ± 0.06	80.1 ± 0.9 ^{a}
80	24	18.8 ± 0.7 ^{a}	0.8 ± 0.04	80.4 ± 0.6 ^{a}
200	19	17.5 ± 0.5 ^{ab}	0.8 ± 0.04	81.7 ± 0.5 ^{ab}
500	23	16.3 ± 0.4 ^{b}	0.8 ± 0.03	82.9 ± 0.4 ^{b}

Table 3.6: Haematocrit, leucocrit and plasma percentages of turbot (*S.maximus*) fed with different levels of vitamin E for 12 weeks. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

The percentage of erythrocytes was significantly higher ($p = 0.001$) in fish fed on depleted or low vitamin E diets compared with fish fed on high vitamin E levels, whereas the percentage of plasma was increased ($p = 0.0025$) in fish fed high vitamin E levels compared with fish fed on depleted or low vitamin E diets. No significant differences in leucocrit were measured between the dietary treatments (Kruskal-Wallis, $p < 0.05$).

3.6.1.2 - Blood smears

Data on the different leucocyte types in blood smears of turbot are presented in Table 3.7. Statistical analysis (Kruskal-Wallis test) showed significant differences in differential counts for phagocytic cells ($p = 0.0001$), lymphocytes ($p = 0.0029$) and thrombocytes ($p = 0.0001$).

The number of phagocytic cells was clearly increased in the fish fed on a vitamin E-free diet compared with the vitamin E supplemented fish. However, fish fed on the 200 mg vitamin E diet showed a relatively high level of phagocytic cells which was mainly due to a high level

of phagocytic cells from fish in tank 3, (one of the duplicates of treatment 3, which had about 30% of phagocytic cells and where an infection occurred).

Higher numbers of lymphocytes were counted in blood from fish fed with 80 or 200 mg of vitamin E compared with fish fed depleted vitamin E diets. In contrast, the number of thrombocytes was decreased in fish fed with 200 mg of vitamin E compared with fish fed on diets containing 80 or 500 mg of vitamin E.

Diet	N	Phagocytes	Lymphocytes	Thrombocytes
0	22	25.3 ± 2^{a}	40.0 ± 3^{a}	34.5 ± 3^{ab}
80	26	7.3 ± 0.9^{b}	51.7 ± 2^{bc}	41.0 ± 3^{ac}
200	19	18 ± 3.5^{c}	53.0 ± 4^{b}	29.0 ± 2^{b}
500	23	9.9 ± 2^{b}	43.8 ± 2^{ac}	45.8 ± 2^{c}

Table 3.7: Differential leucocyte counts in blood from turbot (*S.maximus*) fed for 12 weeks with different levels of vitamin E. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

3.6.1.3 - Phagocytosis assay

The data are presented as the average ± 1 SE for kidney (Fig. 3.2) and for spleen (Fig. 3.3). Seven fish were sampled for diet 1, ten for diet 2, seven for diet 3 and nine for diet 4 for the kidney whereas nine, thirteen, ten, and nine individuals were sampled respectively for the splenic leucocyte study. The statistical analysis using a Kruskal-Wallis test did not detect any significant differences between the dietary treatments in either organ.

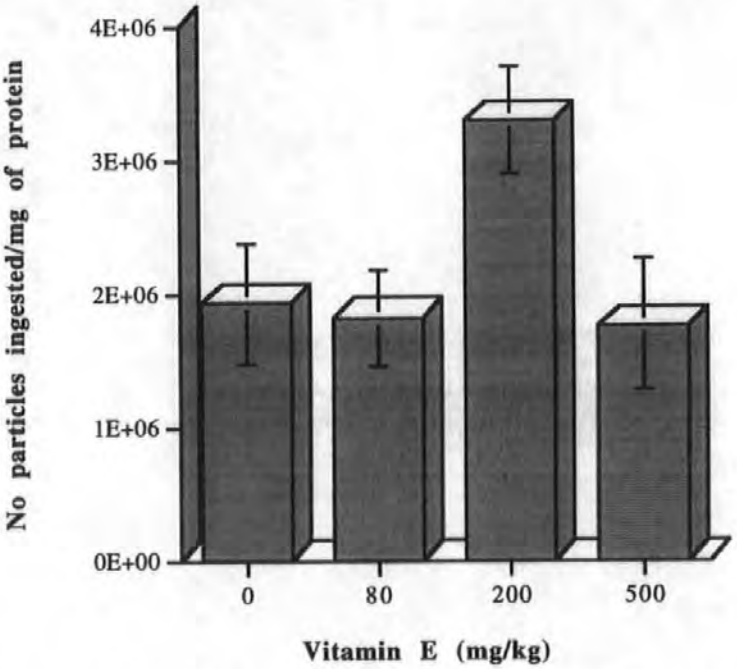


Fig. 3.2: Phagocytosis of dyed zymosan particles by turbot (*S. maximus*) kidney leucocytes after 12 weeks of feeding with different vitamin E levels.

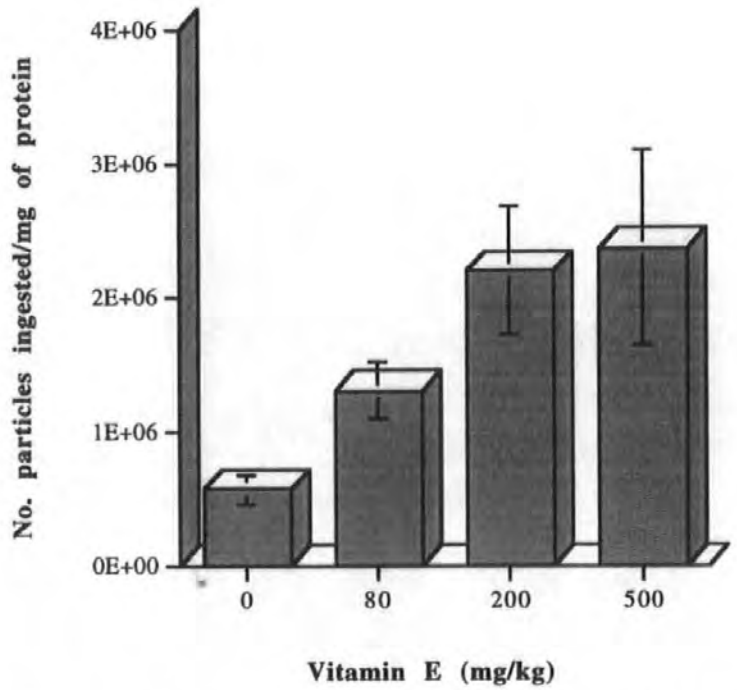


Fig. 3.3: Phagocytosis of dyed zymosan particles by turbot (*S. maximus*) splenic leucocytes after 12 weeks of feeding with different vitamin E levels.

3.6.1.4 - Neutral red uptake assay

The data are presented as the average ± 1 SE for kidney (Fig. 3.4) and for spleen (Fig. 3.5). Eleven fish were sampled for diet 1, fourteen for diet 2, eleven for diet 3 and twelve for diet 4 for the kidney, whereas ten, fourteen, ten, and twelve individuals were sampled respectively for the splenic leucocytes.

The uptake of neutral red was decreased in fish fed with 80 mg of vitamin E compared with fish fed with 200 mg of vitamin E (Kruskal-Wallis, $p = 0.0258$).

The Kruskal-Wallis test failed to show any significant difference in the uptake of neutral red by splenic leucocytes.

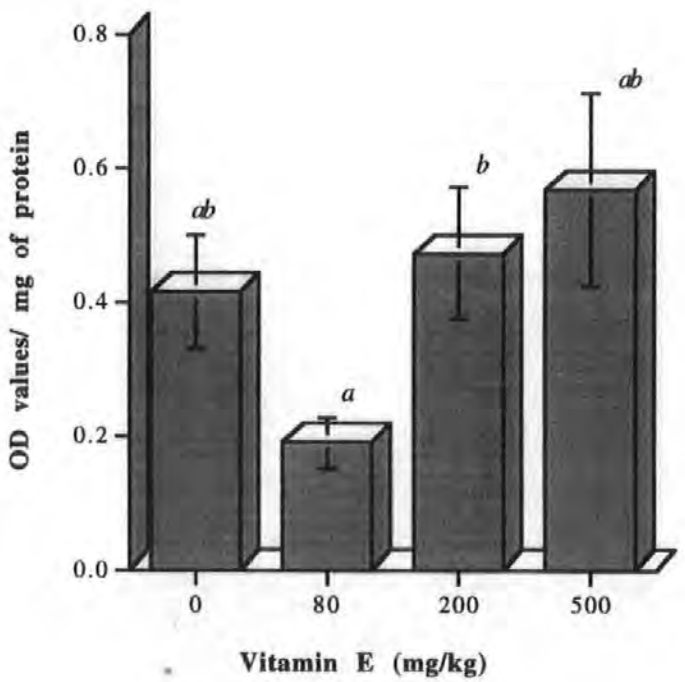


Fig. 3.4: Uptake of neutral red by leucocytes from turbot (*S. maximus*) kidney after feeding for 12 weeks with different levels of vitamin E. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

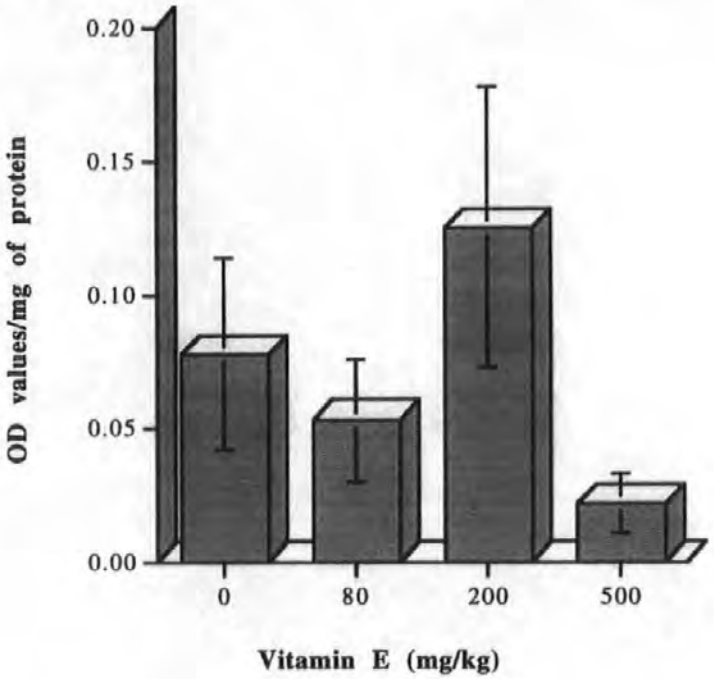


Fig. 3.5: Uptake of neutral red by splenic leucocytes from turbot (*S. maximus*) after 12 weeks feeding with different vitamin E levels

3.6.1.5 - Cell proliferation assay

Lymphocytes sampled from each organ were tested with 3 different mitogens for their ability to proliferate. The data are presented as the average ± 1 SE in Fig. 3.6 (Con A), Fig. 3.7 (LPS) and Fig. 3.8 (PW mitogen). The number of individuals tested for each treatment and each organ is presented in Table 3.8.

Data analysis was carried out between the four dietary treatments for each organ and each mitogen using the Kruskal-Wallis test. No significant difference was detected between the four dietary treatments.

	Con A		LPS		PW mitogen	
Diet	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen
0	11	8	10	8	11	7
80	6	8	8	8	7	6
200	10	10	10	10	9	9
500	6	3	7	4	3	4

Table 3.8: Number of individuals sampled for each test

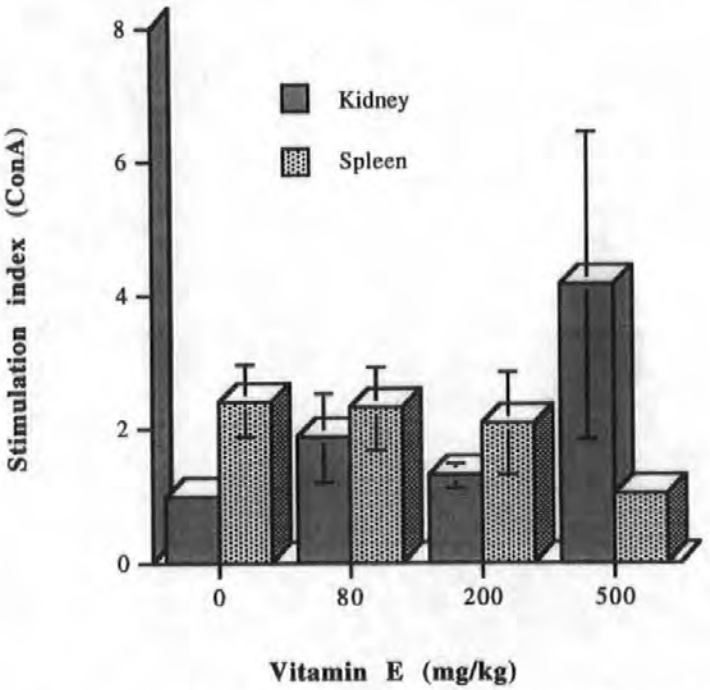


Fig. 3.6: Stimulation indices of kidney and spleen lymphocytes stimulated with ConA

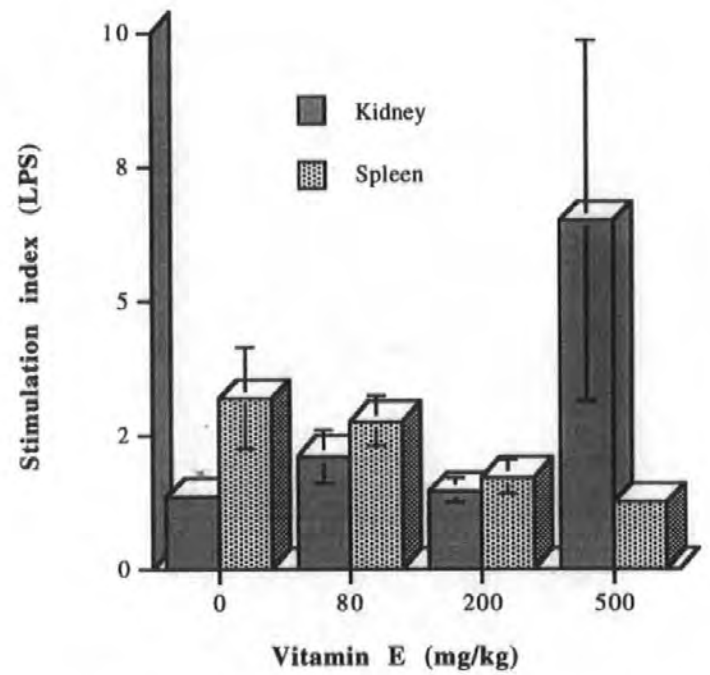


Fig. 3.7: Stimulation indices of kidney and spleen lymphocytes stimulated with LPS

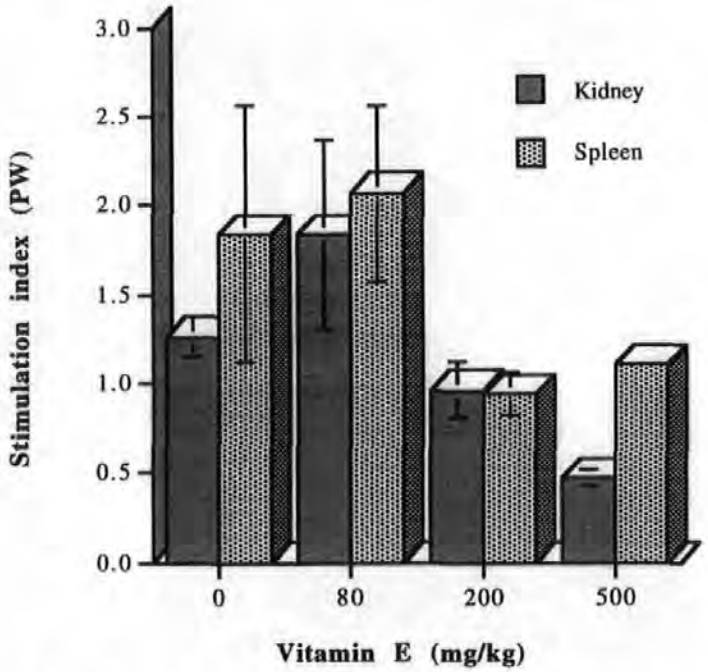


Fig. 3.8: Stimulation indices of kidney and spleen lymphocytes stimulated with PW mitogen

3.62 - Humoral assays

3.6.2.1 - Serum Protein assay

The data are presented as the average ± 1 SE. in Fig. 3.9. Twenty two individuals were tested for diet 1, twenty six for diet 2, nineteen for diet 3, and twenty three for diet 4. The Kruskal-Wallis test carried out on the four groups of fish showed a significant difference between the dietary treatments ($p = 0.001$).

The fish fed with 80 mg of vitamin E demonstrated higher serum protein concentrations than fish fed with depleted or high vitamin E levels and fish fed with high vitamin E levels showed a lower protein concentration than fish fed 200 mg of vitamin E.

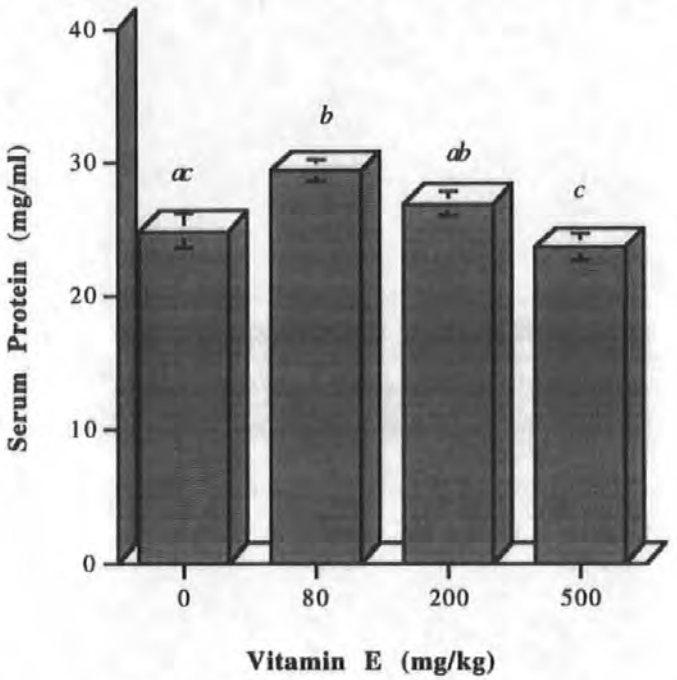


Fig. 3.9: Total serum protein of turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

3.6.2.2 - Lysozyme assay

The data are presented as the average ± 1 SE. in Fig. 3.10. Twenty two individuals were tested for diet 1, 26 for diet 2, 19 for diet 3, and 23 for diet 4. The Kruskal-Wallis test did not show any significant difference between the 4 treatments ($p < 0.05$).

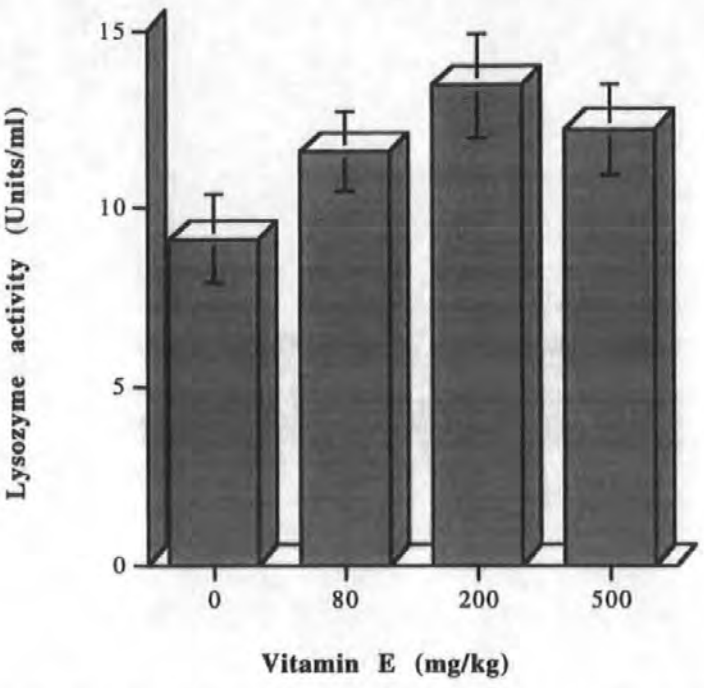


Fig. 3.10: Serum lysozyme activity of turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E

3.7 - FLOW CYTOMETRY

Kidney and spleen cells were separated for the various immunological tests presented previously. The remaining cells, not used in the assays were fixed and subsequently labelled using FITC conjugated PW mitogen and analysed by flow cytometry. Unfortunately no differences could be detected in the FACS profile of different cell populations. Figs. 3.11 and 3.12 show examples of flow cytometry plots of kidney leucocytes.

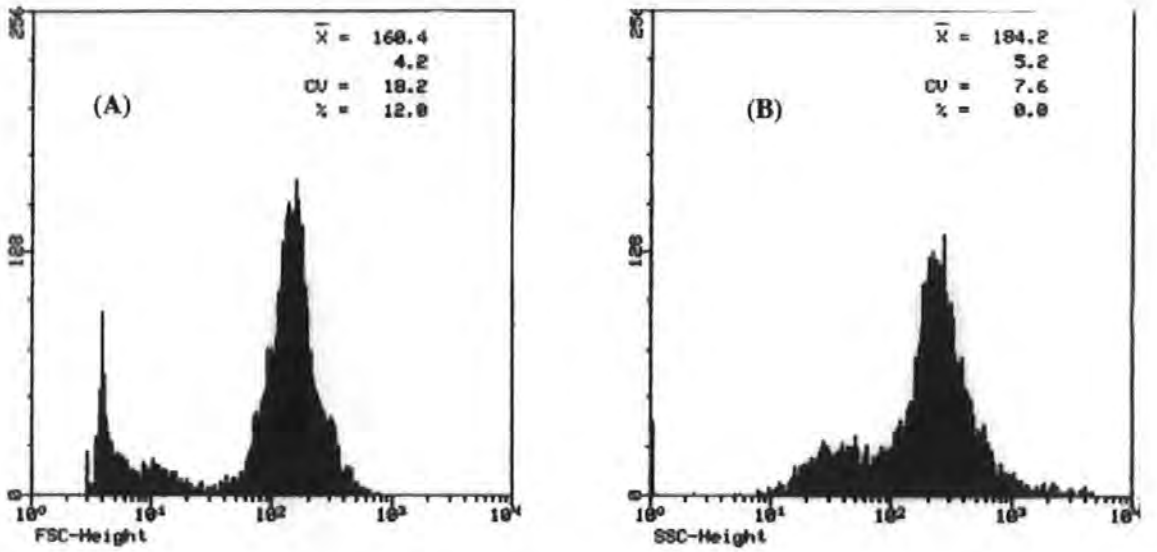


Fig. 3.11: Flow cytometry histogram of anterior kidney cells of turbot (*S. maximus*). The cells were separated on continuous Percoll density gradients. The different layers were run through the flow cytometer. The figure represents the number of events (Y axis) against Forward scatter light (FSC) [A] or against Side scatter (SSC) [B]

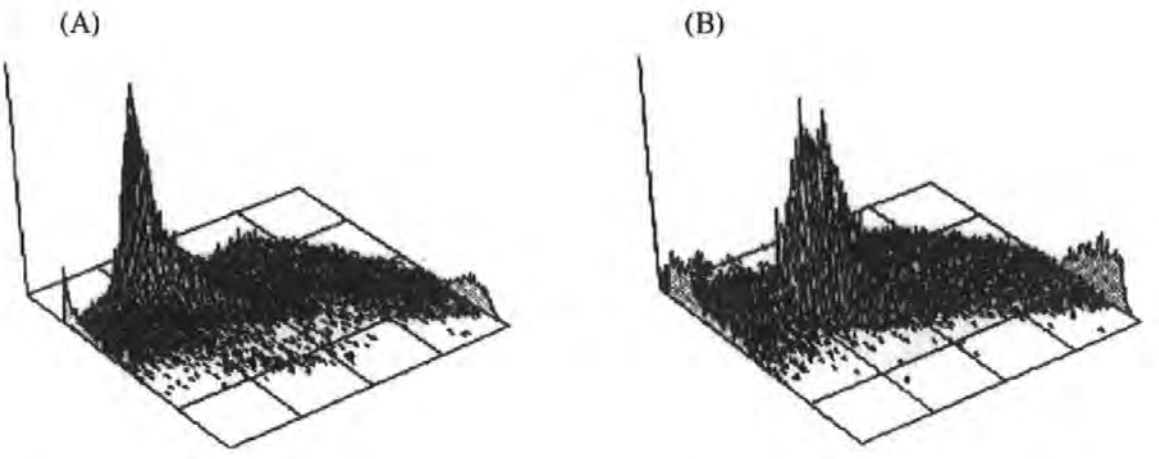


Fig. 3.12: Density plots of anterior kidney cells from fish fed with high vitamin E levels. Cells were incubated with FITC labelled pokeweed mitogen. (A) Forward scatter versus Fluorescence, (B) Side scatter versus Fluorescence

3.8 - DISCUSSION

A summary of the results from the various assays is presented in Table 3.9.

Parameter	0	80	200	500	Statistic
Liver Vitamin E (mg/kg)	11.8 ± 7 ^{a}	58.5 ± 8 ^{b}	178 ± 17 ^{c}	397 ± 44 ^{d}	*p = 0.0001*
Final Length (cm)	13.2 ± 0.3 ^{a}	14.3 ± 0.2 ^{b}	14.3 ± 0.2 ^{b}	13.8 ± 0.3 ^{ab}	*p = 0.014*
Final Weight (g)	34.2 ± 2 ^{a}	45.4 ± 2 ^{b}	47 ± 2 ^{b}	42.4 ± 3 ^{ab}	*p = 0.0015*
Haematocrit (%)	19.5 ± 0.7 ^{a}	18.7 ± 0.6 ^{a}	17.5 ± 0.5 ^{ab}	16.3 ± 0.4 ^{b}	*p = 0.001*
Plasma (%)	80.1 ± 0.8 ^{a}	80.4 ± 0.6 ^{a}	82.7 ± 0.5 ^{ab}	82.9 ± 0.4 ^{b}	*p = 0.0025*
Phagocytic cells (%)	25.3 ± 2.7 ^{a}	7.3 ± 0.9 ^{b}	18 ± 3.5 ^{c}	9.9 ± 1.8 ^{b}	*p = 0.0001*
Lymphocytes (%)	40 ± 3.3 ^{a}	51.7 ± 2.2 ^{bc}	53 ± 3.9 ^{b}	43.8 ± 2.5 ^{ac}	*p = 0.0029*
Thrombocytes (%)	34.5 ± 2.7 ^{ab}	41 ± 2.6 ^{ac}	29 ± 1.9 ^{b}	45.8 ± 2.4 ^{c}	*p = 0.0001*
Neutral red uptake (OD/mg)	0.4 ± 0.08 ^{ab}	0.2 ± 0.04 ^{a}	0.5 ± 0.1 ^{b}	0.6 ± 0.1 ^{ab}	*p = 0.0258*
Protein assay (mg/ml)	24.8 ± 1.2 ^{ac}	29.3 ± 0.8 ^{b}	26.8 ± 0.9 ^{ab}	23.4 ± 1 ^{c}	*p = 0.0001*

Table 3.9 : Summary of results found after feeding turbot (*S.maximus*) for 12 weeks with different level of vitamin E. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

Turbot fed with diets containing different vitamin E levels did not show any differences in their growth rates but their average weights were significantly different at the end of the trial. Several studies carried out on different species including Channel catfish (*Ictalurus punctatus*) (Gatlin *et al.*, 1992; Wise *et al.*, 1993), sea-bass (*Dicentrarchus labrax*) (Messager *et al.*, 1992) and Atlantic salmon (*Salmo salar*) (Hardie *et al.*, 1990) have shown that growth rate is not affected by different dietary levels of vitamin E. The growth rate of turbot fed different levels of vitamin E and vitamin C was also found to be unchanged by dietary treatments (Roberts, unpublished). However, other studies reported that dietary vitamin E depletion could reduce growth of several species. Carp (*Cyprinus carpio*) (Watanabe *et al.*, 1970a,b), trout (*Oncorhynchus mykiss*) (Frischknecht *et al.*, 1994), juvenile Korean Rockfish (*Sebastes schlegeli*) (Bai and Lee, 1998) all showed lower growth when fed vitamin E deprived diets. Finally, Thorarinsson *et al.* (1994) demonstrated an increased average final weight in Chinook salmon fed with an increased level of vitamin E compared with salmon fed on low levels of vitamin E.

In the present experiment, turbot fed on the vitamin E depleted diet had a lower final average weight than turbot fed with 80 or 200 mg of vitamin E, suggesting that a minimum level of vitamin E was necessary to facilitate normal growth. After five weeks, fish deprived of vitamin E became lethargic with a dark skin. These signs extended to all the individuals from the treatment group after seven weeks. Fish developed a problem with vision which impaired visualisation and catching of food pellets. This led to reduced food intake and impaired growth, resulting in a difference in final average weight compared with fish on vitamin E supplemented diets.

A study in which rainbow trout were fed with diets deficient in Vitamin E and vitamin C reported signs of lethargy and dark skin coloration after 8 weeks of feeding (Frischknecht *et al.*, 1994). Anorexia occured and at week 12 all fish were dead.

Incidences of dark coloration, skin ulceration, lethargy, anorexia, emaciation and blindness were also observed in sea bass and other fish cultured in tropical marine conditions by Raymond (1987). These symptoms were described as the blindness melanism syndrome and disappeared when vitamin E and vitamin C were both added to the diet. Histological examinations at the time showed that retinal atrophy was responsible for the blindness.

In the present study, although histological examination of the eye was not carried out, the lesions observed were comparable with those described by Raymond (1987) and could indicate that the problem with vision might originate with a deficiency of antioxidants in the diet. In addition, the diets were supplemented with vitamin C which could explain why the mortalities observed within fish fed with depleted vitamin E diet did not reach 100% as in the study of Frischknecht *et al.*, (1994).

Dietary vitamin E depletion produced a significant increase in haematocrit in the present study whereas other studies on bass (Obach *et al.*, 1993) and rainbow trout (Frischknecht *et al.*, 1994; Furones *et al.*, 1992) have demonstrated a low haematocrit in fish fed with low vitamin E. Juvenile Korean Rockfish showed lower haematocrit when fed vitamin E deprived diets compared with fish fed 20 or 120 mg of vitamin E per kg of diet for 16 weeks. However fish fed on 500 mg of vitamin E had also lower haematocrit than fish fed 20 and 120 mg of vitamin E (Bai and Lee, 1998). Furthermore, Baker and Davies (1996) also reported an increase in haematocrit in catfish fed depleted vitamin E diets. No

differences were found in haematocrit of Atlantic salmon fed different vitamin E levels (Hardie *et al.*, 1990).

In the present study, for all the individuals tested (N = 84) the plasma percentages were negatively correlated with the haematocrit values (R = 0.929, $p < 0.0001$) (high plasma percentage was associated with low haematocrit and *vice versa*). A similar mechanism to that described by Bender (1992) might explain the changes observed although further work should be done to confirm this hypothesis. The author suggested that vitamin E deficient animals could show exudative diathesis, which is a leakage of blood plasma from capillaries into subcutaneous tissues as a result of abnormal permeability of capillary blood vessels. This could cause an apparent increase in the red blood cell percentage within the blood stream. This mechanism is outlined in Fig. 3.13

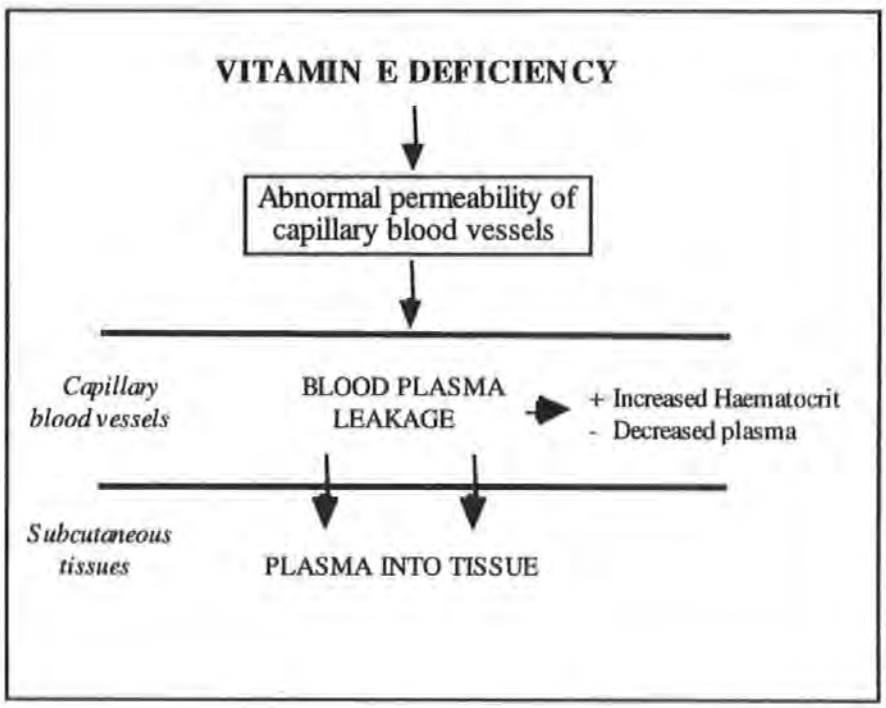


Fig. 3.13: A hypothetical mechanism to explain the haematocrit increase in fish fed a vitamin E deficient diet.

The percentage of phagocytic cells in blood smears from turbot was significantly increased in fish fed on a vitamin E depleted diet compared to the other treatments. Other studies on salmon (Hardie *et al.*, 1990, 1991) and on turbot (Roberts *et al.*, 1995) did not show any significant differences in differential leucocyte counts between groups of fish fed with different levels of vitamin E (Hardie *et al.*, 1990) or vitamin C (Hardie *et al.*, 1991; Roberts *et al.*, 1995). However, two studies on trout and on Atlantic salmon infected with

Renibacterium salmoninarum showed that infected fish had increased numbers of monocytes, thrombocytes and neutrophils (Bruno and Munro, 1986). Neutrophils were increased 2-4 days post-infection in trout and 14 days post infection in salmon, whereas monocytes in both species were increased at day 28 or 35 days post-infection. This may suggest that the increase shown in the present study in the number of phagocytic cells in fish fed on a vitamin E depleted diet might be related to the condition of the fish rather than the dietary vitamin E level. Indeed, fish fed the depleted diet showed signs of infection by the end of the experimental trial. Additionally an increase in the number of phagocytic cells was measured in one of the two groups of fish fed with 200 mg of vitamin E/kg where a disease outbreak occurred.

In the present study the uptake of neutral red by kidney leucocytes was shown to be greater in fish fed with 200 mg compared to fish fed 80 mg of vitamin E but no significant differences were measured between the other groups. Verlhac and Gabaudan (1997) demonstrated an increase in pinocytosis of neutral red by leucocytes of rainbow trout fed with 600 mg of vitamin E for 16 weeks compared with fish fed with 50 mg of vitamin E. More recently two studies in mammals looked at the effect of vitamin E supplements or deficiency on certain physiological processes in lymphocytes and macrophages. Pighetti *et al.* (1998) showed that lymphocytes from rats fed vitamin E and selenium deficient diets were unable to internalise transferrin receptors. It was suggested that this was due to an alteration of endocytosis caused by vitamin E and selenium deficiency. Sakamoto *et al.* (1998) looked at the effect of vitamin E on the secretion of macrophage migration inhibitory factor (MIF) by rat macrophages after intraperitoneal injection of vitamin E. The authors showed that vitamin E inhibited MIF secretion into the culture medium although the cells contained intracellular MIF. It was concluded that MIF secretion was inhibited through modulation of macrophage-membrane architecture by vitamin E. Although the vitamin E content of macrophages was not measured in the present study, the difference in liver vitamin E between fish fed on 80 mg and 200 mg of vitamin E could suggest that there was also a difference in levels in macrophages and this might have played a role in the uptake of neutral red. Modification of the macrophage cell membrane might be responsible for the

increased uptake of neutral red detected in fish fed 200 mg compared with 80 mg although further work would be necessary to test this hypothesis.

The total protein in serum was significantly increased in fish fed 80 mg vitamin E compared with fish fed depleted diets or high vitamin E diets. Decreased serum protein levels have been associated with infections and disease (Yamashita, 1967; Evenberg *et al.*, 1986) and this fits well with the results of the present study where fish fed depleted vitamin E diets have lower total serum protein than fish fed with 80 mg of vitamin E. However, this does not explain the decrease of serum protein in fish fed 500 mg vitamin E. Poston *et al.* (1976) reported that selenium and vitamin E deficiency in salmon gave rise to the appearence of symptoms including exudative diathesis and elevated plasma protein. The author suggested that elevated plasma protein could be the result of liberation of protein from hemolysed erythrocytes and loss of plasma fluid due to exudative diathesis. Given the haematocrit data in the present study haemolysis of red blood cell seems unlikely unless plasma leakage by exudative diathesis was overcoming the loss in red blood cells. However, if we consider the second hypothesis where the increase of plasma protein is associated with exudative diathesis we should expect a correlation between serum total protein and haematocrit. Serum protein and haematocrit are strongly correlated for fish fed with vitamin E supplemented diets ($r = 0.409$, $p < 0.0007$, 66 individuals tested) but the correlation is much weaker when fish fed depleted diet are included in the analysis ($r = 0.264$, $p < 0.0145$, 84 individuals tested). This suggests that although exudative diathesis could be at the origin of the variation in total serum protein between dietary treatments another influence plays a stronger role in the modulation of serum protein in fish fed a depleted diet obscuring the variation caused by exudative diathesis. This could be a defect in protein synthesis caused by liver damage in depleted fed fish.

CHAPTER 4 - EFFECT OF VITAMIN E AND OXIDISED OIL ON THE IMMUNE SYSTEM OF TURBOT (*SCOPHTHALMUS MAXIMUS*)

4.1 - INTRODUCTION

There is evidence that oxidation processes and free radicals may be involved in many pathological conditions such as ageing, because supplying antioxidants to several animal species has been shown to increase their lifespan. It has also been suggested that oxygen radicals are involved in autoimmune diseases. New Zealand Black mice had an increased lifespan and showed decreased manifestations of autoimmune disease when antioxidants, such as tocopheryl acetate or Santoquin, were added to their diet (Harman, 1982).

Oxidative damage has also been reported in other cases where, although not always lethal, some tissue pathology has been induced. Reduced growth, anorexia, pancreas and kidney degeneration and ceroid in the livers were reported in sea-bass (*Dicentrarchus labrax*) farmed in tropical environments and fed on a diet which was rancid and deficient in vitamin E (Gallet de Saint-Aurin, 1987).

Rancidity of the diet is caused by oxidation of the oil, or lipid peroxidation, defined as the oxidative deterioration of polyunsaturated lipids. This may occur during manufacture or storage of food pellets, as the diets are generally prepared using marine oils rich in polyunsaturated fatty acids, which are more prone to peroxidation than saturated fatty acids. Several studies have shown a link between the oxidised fish oils used in diets and pathology (Hashimoto *et al.*, 1966; Watanabe and Hashimoto, 1968; Smith, 1979; Hung *et al.*, 1983; Moccia *et al.*, 1984; Raymond, 1987; Baudin Laurencin *et al.*, 1989; Messager *et al.*, 1992; Obach *et al.*, 1992).

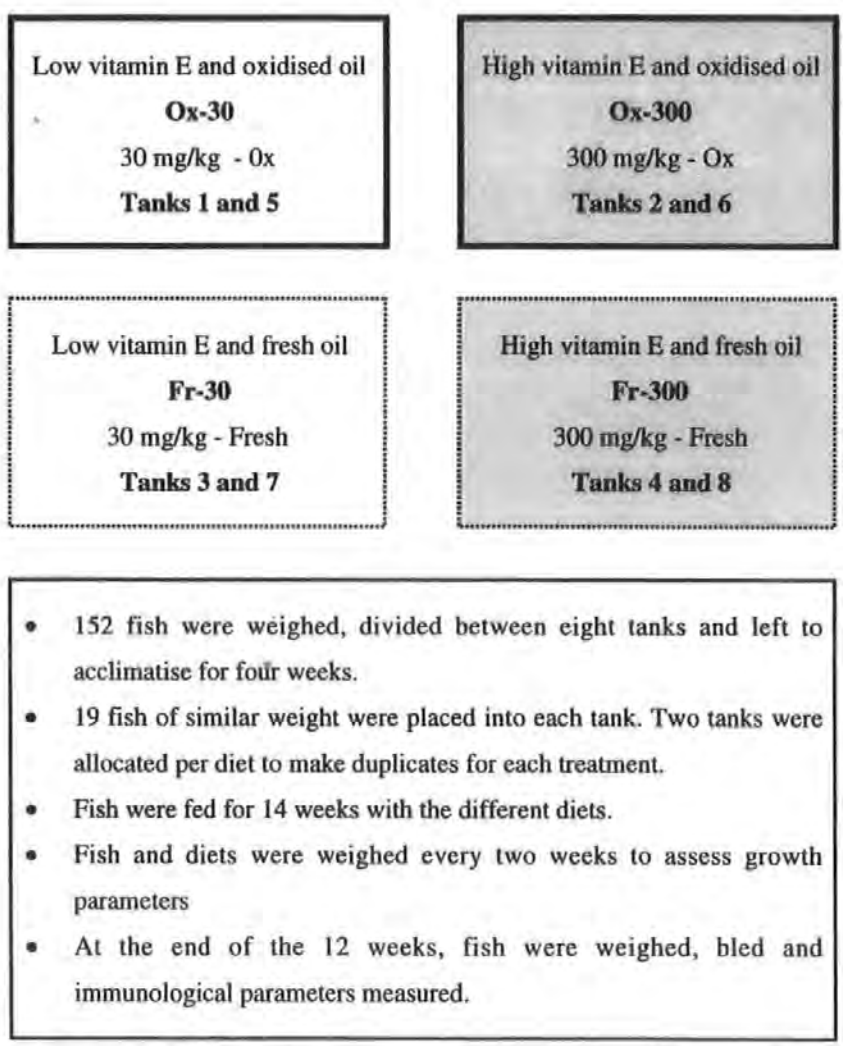
Vitamin E is widely used as a food supplement and has been shown to be essential in the diet of animals. It is the major lipid-soluble antioxidant and therefore is potentially a beneficial additive to prevent pathogenic effects resulting from oxidation of the diet. In fish, deficiency of vitamin E in the diet can compromise immune responsiveness (Blazer and Wolke, 1984; Hardie *et al.*, 1990; Blazer, 1991; Wise *et al.*, 1993; Verlhac and Gabaudan, 1997).

The present study was undertaken to investigate the effects of different levels of oil oxidation and vitamin E in the diet, on a range of non-specific and specific defence mechanisms of turbot (*Scophthalmus maximus*). Juvenile turbot were fed on four different

diets which were supplemented with either oxidised or fresh oil and different levels of α-tocopherol acetate as follows: 1, oxidised oil and 30 mg vitamin E/kg of diet; 2, oxidised oil and 300 mg vitamin E/kg of diet; 3, fresh oil and 30 mg vitamin E/kg of diet; 4, fresh oil and 300 mg vitamin E/kg of diet. The aim was to allow a determination of the effects of (1) dietary oil oxidation on immune responses and (2) the ability of dietary vitamin E to alleviate these effects. In addition data were derived on the effects of different doses of vitamin E on immune parameters.

4.2 - EXPERIMENTAL PROTOCOL

The following figure represents the experimental protocol:



4.3 - COMPOSITION OF THE EXPERIMENTAL DIET

4.3.1 - Chemical composition of the diets

Table 4.1 presents the moisture, ash, protein and lipid composition of the diets. Diets were composed of approximately 49% protein, 9% lipids and 13% ash. These diets were high in protein content but lower than expected in lipids, as 10% of lipid (in the form of cod liver oil) was added to the diets. A lipid content in excess of 10% was expected as lipids were also present in other ingredients such as fish meal.

Diet	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
Ox-30	7.8	12.7	50.1	9.9
Ox-300	8.0	13.1	48.6	9.9
Fr-30	9.8	13.0	49.0	8.7
Fr-300	10.8	13.1	48.0	7.2

Table 4.1: Composition of practical test diets expressed as percentage of wet weight

4.3.2 - Analysis of vitamin E levels

Table 4.2 shows the levels of vitamin E in the diets after manufacture measured by high-performance liquid chromatography (HPLC) at Roche (St Louis, France). The data presented are the mean of 2 replicates.

Diet	Included (mg/kg)	Measured (mg/kg)
Ox-30	30	16
Ox-300	300	158
Fr-30	30	24
Fr-300	300	250

Table 4.2: Levels of vitamin E in the diets before and after processing (mg vitamin E/kg of diet)

The results show loss of detectable vitamin E in diets prepared with oxidised oil compared with the fresh oil diets prepared with the same levels of vitamin E. Nevertheless a general loss of detectable vitamin E during feed processing was seen in all of the diets.

4.3.3 - Lipid analysis

Lipid analysis was carried out by gas chromatography (GC) using a marinol standard as a reference to identify the peaks. The GC trace of the marinol standard is shown in Fig.4.1 and a representative GC trace from an experimental sample is shown in Fig. 4.2. The lipid composition of the diets is shown in Table 4.3 and the values presented are the means of three samples ± 1 SE.

Statistical analysis using the Kruskal-Wallis test only revealed a significant difference between the 4 diets for the 18:3 (n-6) composition. The level of 18:3 (n-6) was highest in livers of fish fed fresh oil and high vitamin E and lowest in fish fed with low vitamin E and oxidised oil. No significant differences were characterised for the sum of saturated, monounsaturated, PUFAs, total (n-3), total (n-6) or (n-3)/(n-6) ratio between the different treatments.

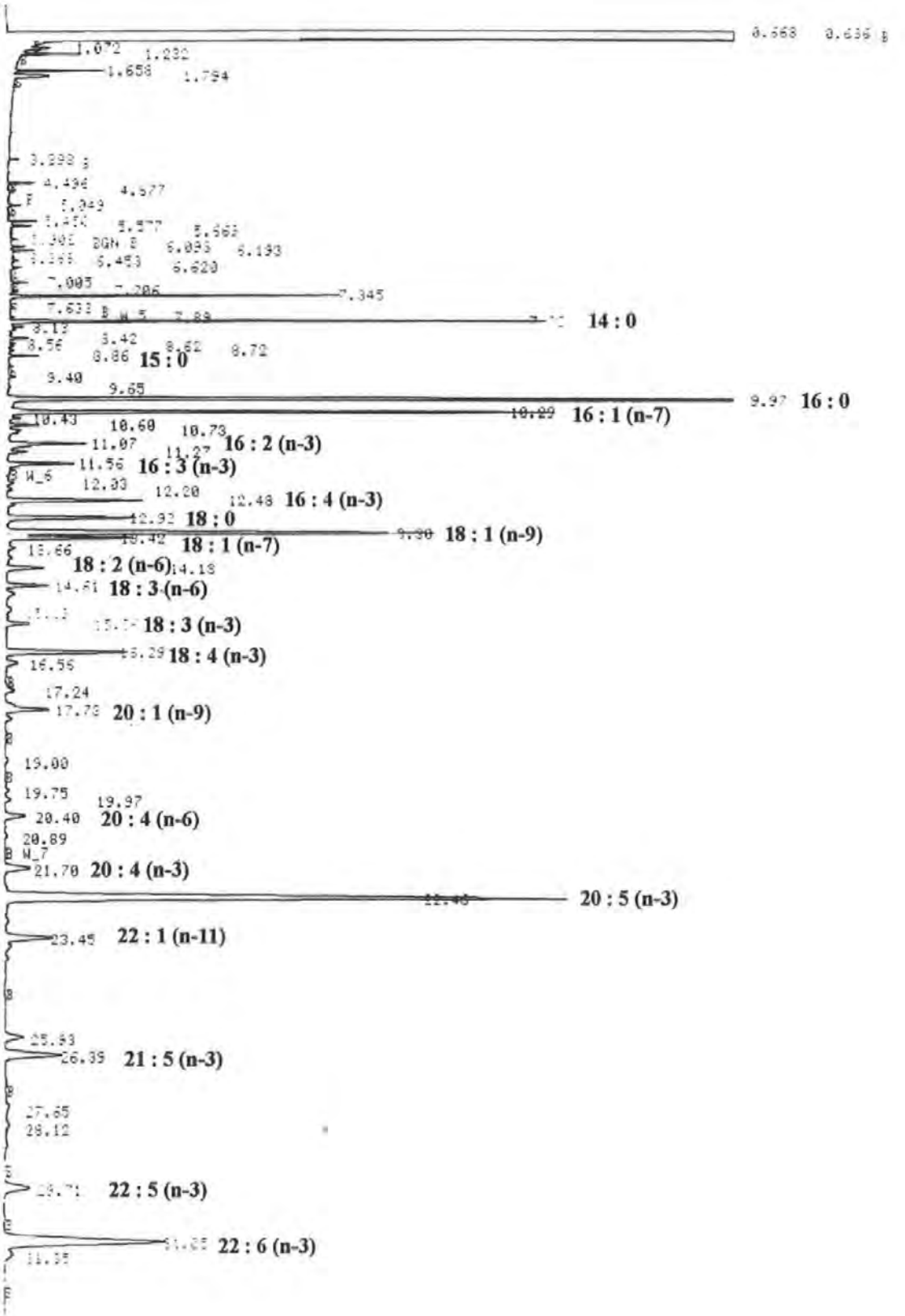


Fig. 4.1: Gas chromatography trace of marinol standard used as reference for fatty acid determination

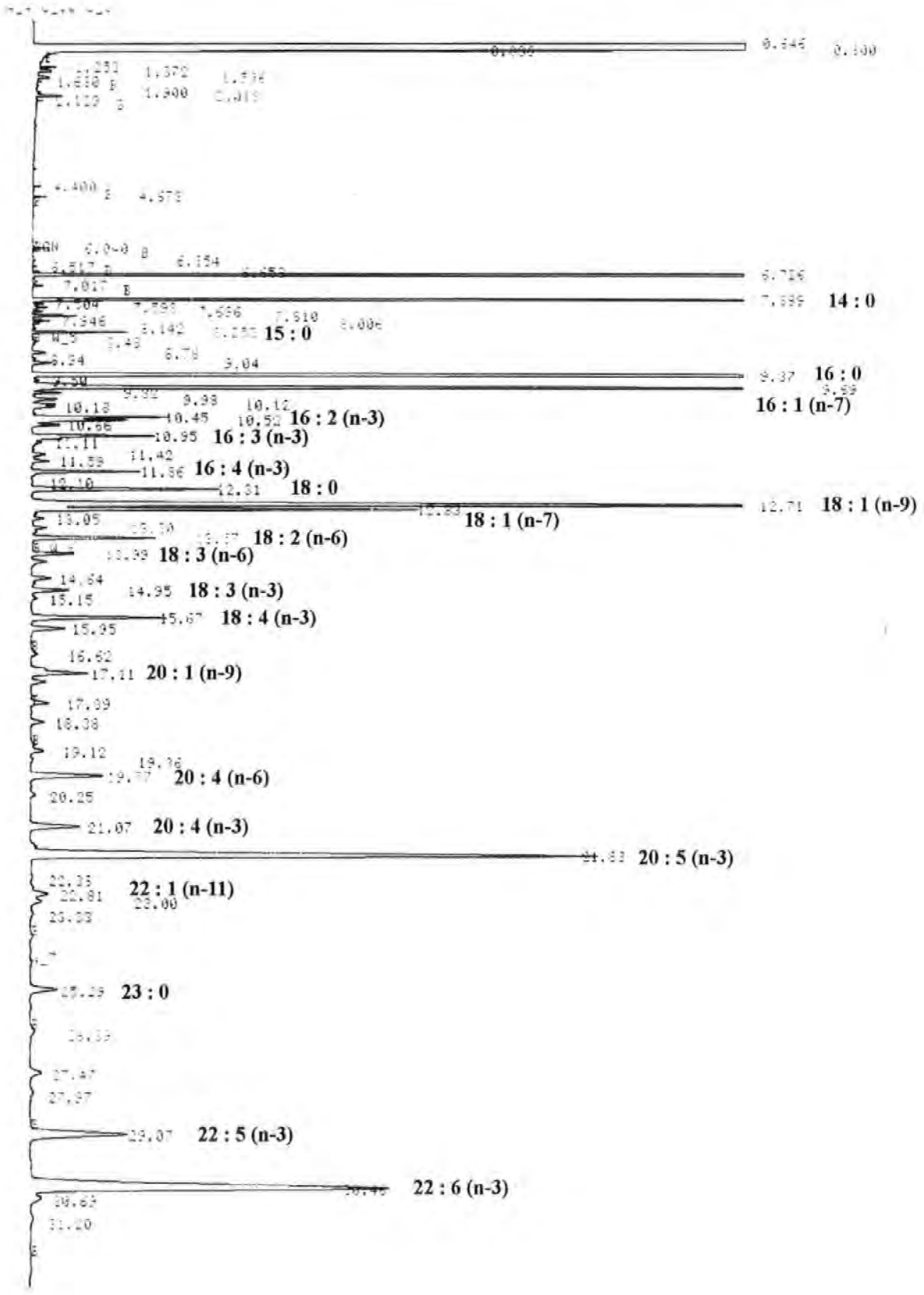


Fig. 4.2: Gas chromatography trace of lipid extracted from the liver of turbot (*S. maximus*) fed with FR-300 diet

Fatty acid	Ox-30	Ox-300	Fr-30	Fr-300	Statistics
14:0	11.1 ±0.8	10.5 ±0.3	10.1 ±1	8.6 ±0.5	
15:0	0.8 ±0.06	0.8 ±0.02	0.8 ±0.07	0.6 ±0.03	
16:0	29.5 ±2.2	27.8 ±1	26.5 ±2	22.5 ±0.4	
16:1 (n-7)	11.4 ±0.4	11 ±0.07	11.8 ±0.9	10.5 ±0.4	
16:2 (n-3)	0.6 ±0.3	0.6 ±0.08	0.9 ±0.1	1.1 ±0.04	
16:3 (n-3)	0.5 ±0.26	0.7 ±0.09	0.9 ±0.3	1.2 ±0.2	
16:4 (n-3)	0.8 ±0.34	1 ±0.2	1 ±0.3	1.6 ±0.1	
18:0	7.5 ±0.8	7.1 ±0.2	7.1 ±0.6	6.2 ±0.3	
18:1 (n-9)	15.7 ±1	14.9 ±0.2	16.2 ±1.3	14.3 ±0.6	
18:1 (n-7)	4.9 ±0.4	4.5 ±0.09	4.9 ±0.35	4.4 ±0.09	
18:2 (n-6)	0.7 ±0.1	0.8 ±0.04	1 ±0.1	1.2 ±0.05	
18:3 (n-6)	0.2 ±0.05	0.3 ±0.006	0.4 ±0.02	0.5 ±0.008	*p = 0.0273*
18:3 (n-3)	0.1 ±0.13	0.4 ±0.008	0.3 ±0.1	0.5 ±0.02	
18:4 (n-3)	0.6 ±0.5	1.2 ±0.1	1.1 ±0.7	1.8 ±0.27	
20:0	0.4 ±0.03	0.4 ±0.006	0.4 ±0.03	0.2 ±0.12	
20:1 (n-9)	2.3 ±0.2	2.1 ±0.08	2.4 ±0.2	2.1 ±0.09	
20:4 (n-6)	0.3 ±0.15	0.4 ±0.04	0.5 ±0.17	0.7 ±0.03	
20:4 (n-3)	0.2 ±0.1	0.4 ±0.04	0.3 ±0.13	0.5 ±0.05	
20:5 (n-3)	5.6 ±2.5	7.1 ±0.9	6.3 ±2.7	11.1 ±1	
22:1 (n-11)	1.1 ±0.1	1.1 ±0.05	1.3 ±0.07	1.1 ±0.04	
22:5 (n-3)	0.7 ±0.3	0.9 ±0.1	0.9 ±0.4	1.4 ±0.1	
22:6 (n-3)	3.9 ±1.6	5.1 ±0.3	4.2 ±1.8	7.2 ±0.7	
24:1 (n-9)	0.9 ±0.08	0.8 ±0.04	0.9 ±0.04	0.7 ±0.03	
Saturated	49.3 ±3.8	46.6 ±1.6	44.9 ±6.4	38.2 ±1.4	
Monounsaturated	36.4 ±1.9	34.4 ±0.4	37.4 ±2.8	33.1 ±1.2	
PUFAs	14.3 ±5.7	18.9 ±1.9	17.6 ±6.5	28.7 ±2.6	
Total (n-3)	13 ±5.4	17.4 ±1.9	15.8 ±6.3	26.4 ±2.5	
Total (n-6)	1.3 ±0.3	1.5 ±0.08	1.9 ±0.3	2.3 ±0.09	
(n-3)/(n-6)	9.1 ±2	11.3 ±0.6	7.8 ±2.4	11.2 ±0.6	

Table 4.3: Fatty acid composition of diets. PUFAs for which significant differences were measured are highlighted and the corresponding p values are presented in the right column

4.4 - UPTAKE OF NUTRIENT

4.4.1 - Liver vitamin E

The liver vitamin E content of fish after feeding for 14 weeks with the different diets are shown in Fig. 4.3. The data are the mean ± 1 SE for 9 individuals for Ox-30, 10 individuals for Ox-300 and Fr-30, and 11 individuals for the Fr-300 group. Statistical analysis showed a significant difference between the four dietary treatments using the Kruskal-Wallis test ($p = 0.0001$) and a good correlation between the levels of vitamin E in the diet and the levels measured in the livers ($r = 0.63$, $p = 0.01$).

Although fish fed on Ox-30 and Ox-300 were fed with two different levels of vitamin E, the levels of vitamin E measured in the livers of these two groups were not significantly different. On the other hand the levels of vitamin E found in livers of fish fed on Fr-30 and Ox-30 were significantly different although the levels in the diet should be identical. Fish fed on Fr-300 accumulated higher levels of vitamin E in their livers than fish fed on Fr-30 and Ox-30.

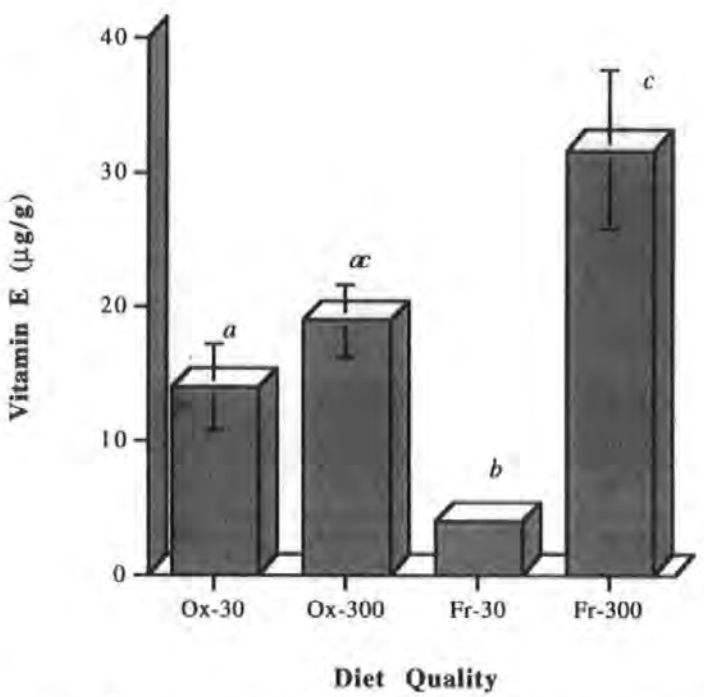


Fig. 4.3: Vitamin E levels in livers of turbot (*S. maximus*) fed for 14 weeks with different vitamin E levels and either oxidised or fresh oil. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.4.2 - Fatty acid composition of the livers

The liver fatty acid compositions are shown in Table 4.4.

Fatty acid	Ox-300	Fr-30	Fr-300	Statistics
14:0	4.3 ±0.8 ^{a}	6.2 ±0.5 ^{a}	8.1 ±0.5 ^{b}	*p = 0.0218*
15:0	0.5 ±0.01	0.5 ±0.01	0.6 ±0.02	
16:0	17.3 ±1.5	17.3 ±0.3	16.9 ±0.4	
16:1 (n-7)	5.4 ±1.2 ^{a}	9.5 ±0.6 ^{b}	11.5 ±0.4 ^{c}	*p = 0.013*
16:2 (n-3)	0.6 ±0.13 ^{a}	0.9 ±0.09 ^{a}	1.3 ±0.06 ^{b}	*p = 0.015*
16:3 (n-3)	7.9 ±7.4	0.9 ±0.16	1.4 ±0.09	
16:4 (n-3)	0.4 ±0.05 ^{a}	0.9 ±0.17 ^{ab}	1.2 ±0.1 ^{b}	*p = 0.0228*
18:0	4.9 ±0.4 ^{a}	3.1 ±0.3 ^{b}	2.3 ±0.09 ^{b}	*p = 0.0125*
18:1 (n-9)	8.3 ±1.2 ^{a}	11.6 ±0.3 ^{b}	11.7 ±0.3 ^{b}	*p =0.0241*
18:1 (n-7)	3.9 ±0.3	4.1 ±0.04	4.2 ±0.1	
18:2 (n-6)	1.2 ±0.4	1.5 ±0.1	1.6 ±0.03	
18:3 (n-6)	0.4 ±0.12	0.6 ±0.03	0.6 ±0.03	
18:3 (n-3)	0.2 ±0.08 ^{a}	0.5 ±0.03 ^{b}	0.6 ±0.02 ^{b}	*p = 0.0154*
18:4 (n-3)	0.8 ±0.17 ^{a}	1.8 ±0.2 ^{b}	2.1 ±0.09 ^{b}	*p = 0.0125*
20:0	0.2 ±0.06	0.2 ±0.009	0.2 ±0.004	
20:1 (n-9)	1.3 ±0.2	1.1 ±0.1	1.1 ±0.06	
20:4 (n-6)	2.9 ±0.3 ^{a}	1.7 ±0.2 ^{b}	1.4 ±0.08 ^{b}	*p = 0.0183*
20:4 (n-3)	1.1 ±0.6	0.9 ±0.04	1.1 ±0.03	
20:5 (n-3)	11.1 ±1.3 ^{a}	15.6 ±0.7 ^{b}	14.3 ±0.6 ^{ab}	*p = 0.0296*
22:1 (n-11)	3.2 ±2.8	0.4 ±0.03	0.5 ±0.03	
22:5 (n-3)	2.8 ±0.25	3.2 ±0.2	3.2 ±0.09	
22:6 (n-3)	20.8 ±2.4	17.3 ±1.6	14.2 ±1	
24:1 (n-9)	0.5 ±0.3	0.1 ±0.1	0.2 ±0.1	
Sats	27.2 ±4	27.3 ±0.16	28 ±0.7	
Monos	22.5 ±4.4	26.8 ±0.7	29.2 ±0.8	
PUFAs	50.3 ±5.4	45.8 ±0.6	42.8 ±1.4	
Total (n-3)	45.7 ±5.7	42 ±0.6	39.2 ±1.3	
Total (n-6)	4.5 ±0.6	3.8 ±0.1	3.6 ±0.06	
(n-3)/(n-6)	11.1 ±2.7	11.1 ±0.5	11 ±0.3	

Table 4.4 : Fatty acid composition of turbot (*S. maximus*) livers. PUFAs for which significant differences were characterised are highlighted and corresponding p values are presented in the right column. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

Livers were analysed by GC for fatty acid composition and the results are expressed as the mean of 4 individuals ± 1 S.E.

Mortalities in fish fed with oxidised oil and low vitamin E reduced the number of individuals and consequently the number of livers available at the end of the feeding period. All the livers from surviving fish fed Ox-30 were used for vitamin E determination and as a result no livers were available for GC analysis.

Although no significant differences could be characterised for the percentages of total PUFAs, total (n-3) PUFAs or total (n-6) PUFAs, some statistical differences could be detected for several PUFAs between livers of animal fed different diets. Amongst the saturated fatty acids, 18:0 was increased in fish fed with oxidised oil compared with fish fed with fresh oil, whereas 14:0 was increased in fish fed with fresh oil and high vitamin E compared with other treatments. Amongst the PUFAs, 18:1 (n-9), 18:3 (n-3) and 18:4 (n-3) were increased in livers of fresh oil-fed fish compared with livers of oxidised oil-fed fish. The level of 16:1 (n-7) in livers differed between all the treatments tested and increased with both vitamin E and fresh oil. The content in 16:4 (n-3) was increased in livers of fish fed Fr-300 compared with livers of fish fed Ox-300. Finally, 20:5 (n-3) liver content was increased in fish fed Fr-30 compared with fish fed Ox-300 but no differences were noted between livers of Ox-300 fed fish and those of fish fed with Fr-300 diets. Unlike the other PUFAs, 20:4 (n-6) was found in higher proportion in livers from fish fed with oxidised oil than in livers of fish fed with fresh oil.

4.5 - GROWTH PERFORMANCE

4.5.1 - Growth

Data showing the average weights of fish from different treatments during the 14 week feeding period are shown in Fig. 4.4 while the final mean weight and length are presented in Table 4.5. Values presented are the mean of N individuals (as specified in the table) ± 1 SE. Fig. 4.4 shows standard error bars for the beginning and the end of the experiment when the fish were all weighed individually.

From the first day of the trial, fish fed on oxidised oil diets did not feed well. To measure the influence of diet upon immune function it was necessary to encourage the fish to feed and a reduction of the oxidation level of the diet was implemented at week 5 as follows. Fifty per cent of diet 3 was mixed with 50% of diet 1 and this mixture was substituted for diet 1 and 50% of diet 4 was mixed with 50% of diet 2 and this mixture substituted for diet 2. Therefore, the included level of vitamin E was unchanged from the original diet but the level of oxidised oil was reduced by half so the fish were more likely to accept the diet which nevertheless remained oxidised.

Statistical analysis using a Kruskal-Wallis test revealed a significant difference between the different dietary groups for both the final weight ($p = 0.0001$) and the final length ($p = 0.0002$). Different superscript letters in the table indicate a significant difference.

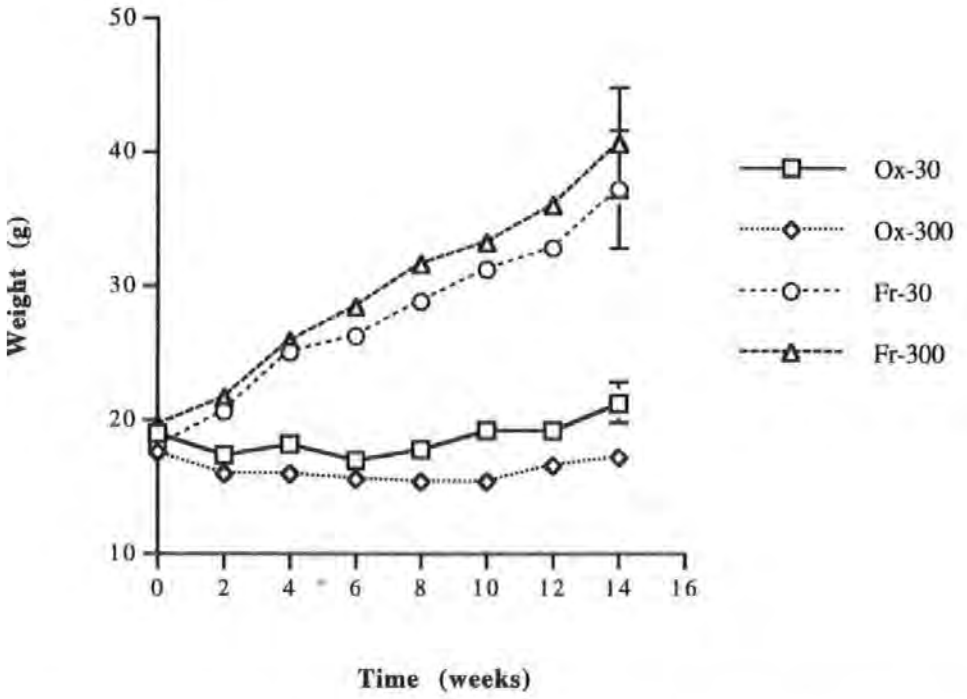


Fig 4.4: Weight recordings from turbot (*S. maximus*) during the 14 weeks of feeding with various levels of vitamin E and either oxidised or fresh oil

Diet	N	Weight (g)	Length (cm)
Ox-30	9	16.8 ± 1.4^{ab}	10.7 ± 0.3^{ab}
Ox-300	16	13.0 ± 1^{a}	10.0 ± 0.2^{a}
Fr-30	21	31.0 ± 3.8^{bc}	12.0 ± 0.5^{bc}
Fr-300	19	37.0 ± 3.6^{c}	12.8 ± 0.5^{c}

Table 4.5: Final average weight and length of turbot (*S. maximus*). Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.5.2 - Specific Growth Rate (SGR)

The cumulative SGR and FCR of turbot fed the different diets are shown in Table 4.6. The data are presented as the mean of the two replicates ± 1 SE as the FCR and SGR for each individual fish could not be calculated due to the impractibility of tagging the individuals and recording individual weights. N represents the total number of individuals (sum of the two replicates) for each treatment.

The statistical analysis carried out on SGR data failed to show a significant difference between the four groups of fish (Kruskal-Wallis test, $p < 0.05$).

Diet	N	SGR	FCR
Ox-30	9	-0.7 ± 0.07	-1.9 ± 0.07
Ox-300	13	-0.3 ± 0.2	-5.2 ± 2.2
Fr-30	13	0.7 ± 0.01	1.9 ± 0.08
Fr-300	18	0.7 ± 0.05	2.1 ± 0.09

Table 4.6: Cumulative FCR and SGR from turbot (*S. maximus*) fed with different levels of vitamin E and either oxidised or fresh oil

4.5.3 - Feed Conversion Ratio (FCR)

Data are presented in Table 4.6. Statistical analysis did not demonstrate any differences between the four groups of fish (Kruskal-Wallis test, $p < 0.05$)).

4.5.4 - Behavioural recordings

As soon as the feeding trial started the fish fed on the diets containing oxidised oil showed some feeding difficulties; pellets caught by the fish were generally rejected and soon the schooling behaviour of coming to the surface to feed also seemed affected. Whereas the fish fed on the diet containing fresh oil showed an increasing appetite, the fish fed with oxidised oil regurgitated the food. After 4 weeks of the feeding period it was decided to make a mixture of diets to encourage fish from the treatments 1 and 2, who were not feeding and consequently losing weight, to feed. The level of oxidation of the diet was reduced by half while the level of included vitamin E was unchanged. The fish fed with this mixture started to eat and showed some signs of growth although both their growth and appetite remained reduced compared with the fish fed with fresh oil diets.

At week 10 fish fed with the partially oxidised diet showed signs of infection characterised by pink coloration at the margins of the body, including the fins, and particularly on the tail which in some cases was eroded before the fish died. Several fish from the oxidised treatments showing those symptoms died.

The cumulative mortality over the 14 weeks of feeding was calculated and is presented in Fig. 4.5. The fish fed diets containing oxidised oil showed a markedly higher mortality than fish fed with diets containing fresh oil. The only case of mortality detected in fish fed with fresh oil was due to an inability of one individual to feed after breaking its jaw. Amongst oxidised oil fed fish the mortality was markedly increased when fish were fed with lower vitamin E levels as opposed to higher vitamin E supplementation (>60% vs 26%).

In the present study a change in skin pigmentation was also detected; some of the turbot fed with depleted vitamin E and oxidised oil showed signs of melanism.

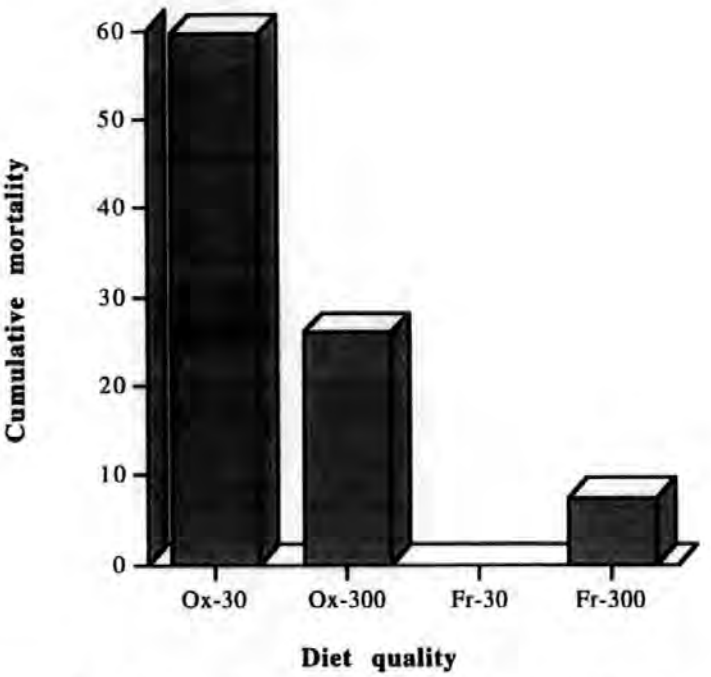


Fig 4.5: Cumulative mortality of turbot (*S. maximus*) fed for 14 weeks with different levels of vitamin E and either oxidised or fresh oil

4.6 - ASSAYS FOR IMMUNOCOMPETENCE

4.6.1 - Cellular

4.6.1.1 - Haematocrit

The data presented in Table 4.7 show the different percentages of red blood cells, white blood cells and plasma in turbot blood. The results are expressed as the average of N individuals ± 1 SE.

Statistical analysis using the Kruskal-Wallis test did not show any differences for $p < 0.05$ between the 4 dietary treatments.

Diet	N	Haematocrit (%)	Leucocrit (%)	Plasma (%)
Ox-30	9	16.9 ± 0.9	0.7 ± 0.1	82.3 ± 0.9
Ox-300	11	18.4 ± 1.3	1.0 ± 0.3	80.6 ± 1.3
Fr-30	16	18.4 ± 0.8	0.7 ± 0.1	80.9 ± 0.8
Fr-300	14	18.2 ± 0.8	0.6 ± 0.1	81.9 ± 0.9

Table 4.7: Haematocrit, leucocrit and plasma of turbot (*S. maximus*) after 14 weeks of feeding

4.6.1.2 - Blood smears

Percentages of phagocytes, lymphocytes and thrombocytes are presented in Table 4.8 as the mean of N individuals ± 1 SE.

The Kruskal-Wallis test showed a significant difference between the four dietary treatments ($p < 0.001$) for all the different cell types.

Fish fed on the oxidised oil diets showed a higher percentage of phagocytic cells whereas fish fed on fresh oil diets showed higher percentages of lymphocytes and thrombocytes. It is noteworthy that the thrombocyte population was increased to a greater extent in fish fed with fresh oil and high vitamin E levels compared with the other treatments.

Diet	N	Phagocytes	Lymphocytes	Thrombocytes
Ox-30	9	68 ± 3.2^{a}	12 ± 3.1^{a}	20 ± 2.4^{a}
Ox-300	13	67 ± 4.9^{a}	11 ± 2.2^{a}	21 ± 3.5^{a}
Fr-30	13	29 ± 2.6^{b}	28 ± 2.3^{b}	43 ± 3.4^{b}
Fr-300	18	22 ± 2.6^{b}	22 ± 1.5^{b}	55 ± 2.2^{c}

Table 4.8: White blood cells percentages of turbot (*S. maximus*) blood smears after 14 weeks of feeding. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.6.1.3 - Phagocytosis

Data are presented in Fig. 4.6 as the average of 5 individuals for oxidised oil treatments and 10 individuals for fresh oil treatments, ± 1 SE. The Kruskal-Wallis test performed on the four groups of fish showed a significant difference ($p = 0.048$).

The phagocytosis of zymosan particles was increased in fish fed the Ox-300 diet compared with fish fed with the Fr-30 diet.

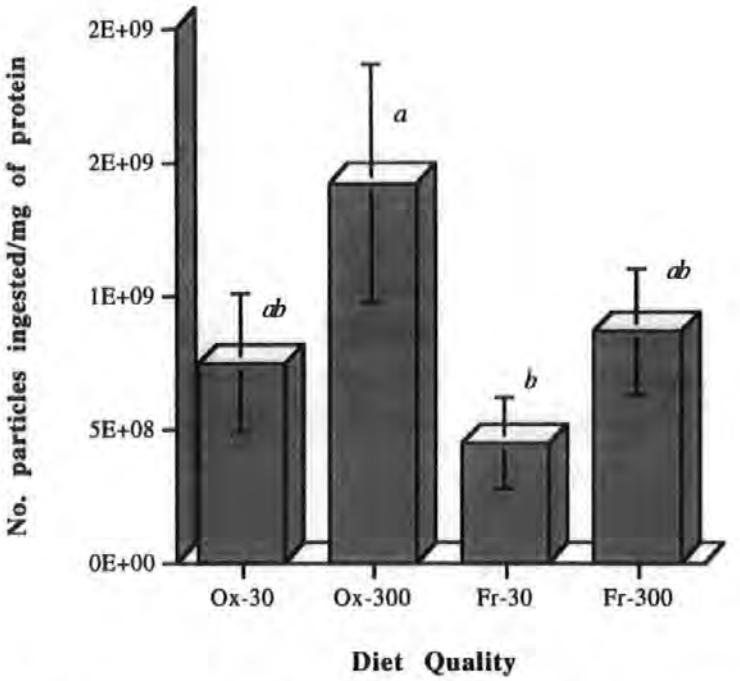


Fig. 4.6: Phagocytosis by kidney leucocytes taken from turbot (*S. maximus*) fed for 14 weeks with different levels of vitamin E and either oxidised or fresh oil. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.6.1.4 - Uptake of neutral red

The uptake of neutral red, expressed as optical density per mg of protein is presented in Fig. 4.7 as the mean of all individuals tested ± 1 SE. Four fish were sampled from the Ox-30 treatment, 7 from 0x-300, 9 from Fr-30 and 8 fish from the Fr-300 treatment. The Kruskal-Wallis test showed a significant difference between the dietary treatments ($p = 0.0033$).

The uptake of neutral red by kidney leucocytes was increased in fish fed with fresh oil diets compared with fish fed on the oxidised oil and high vitamin E diet.

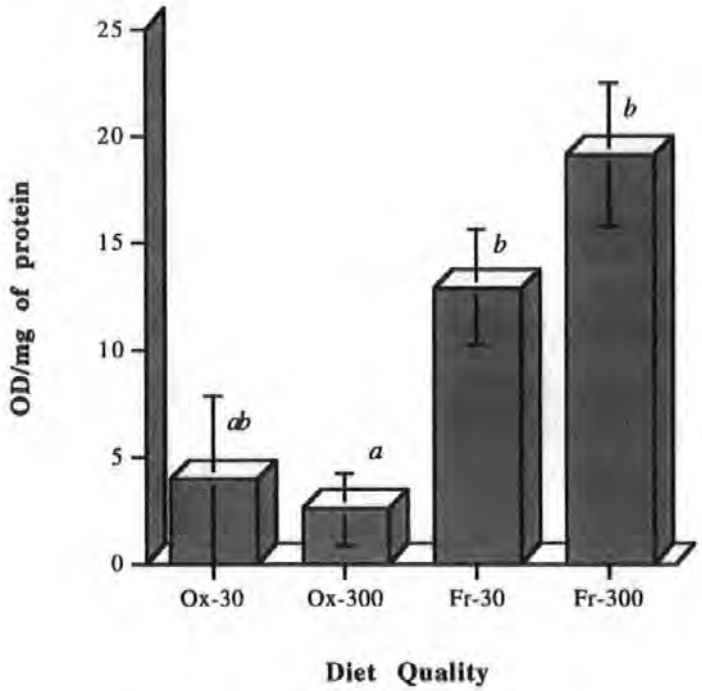


Fig. 4.7: Uptake of neutral red by kidney leucocytes from turbot (*S. maximus*) fed for 14 weeks with different levels of vitamin E and either fresh or oxidised oil. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.6.1.5 - Cell proliferation assay.

The results, expressed as stimulation indices (SI) are shown in Fig. 4.8 for Con A stimulation, Fig. 4.9 for PW mitogen stimulation and Fig. 4.10 for LPS stimulation. Five fish were sampled from the Ox-30 treatment, nine from 0x-300, 11 from Fr-30 and five fish from the Fr-300 treatment.

The Kruskal-Wallis test applied to the data did not show any significant differences between the dietary treatments with any mitogen.

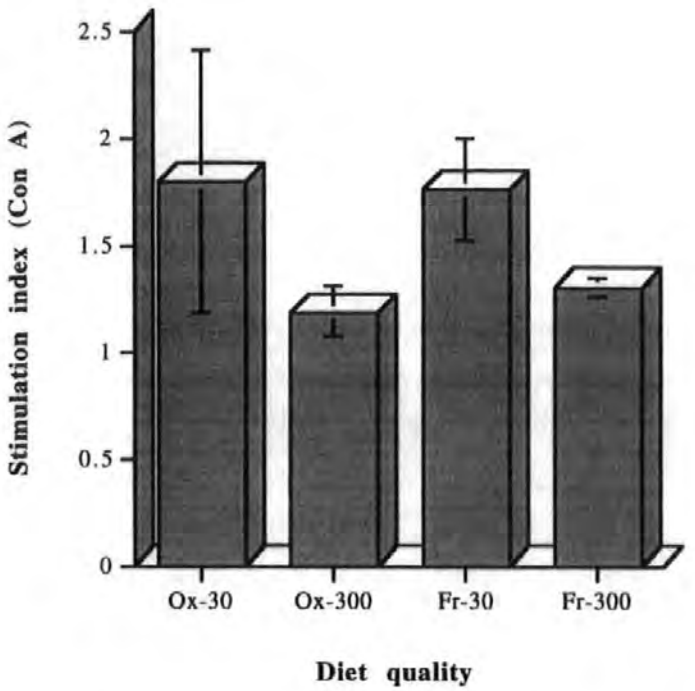


Fig. 4.8: Stimulation Indices (SI) of kidney lymphocytes from turbot (*S. maximus*) stimulated with Concanavalin A

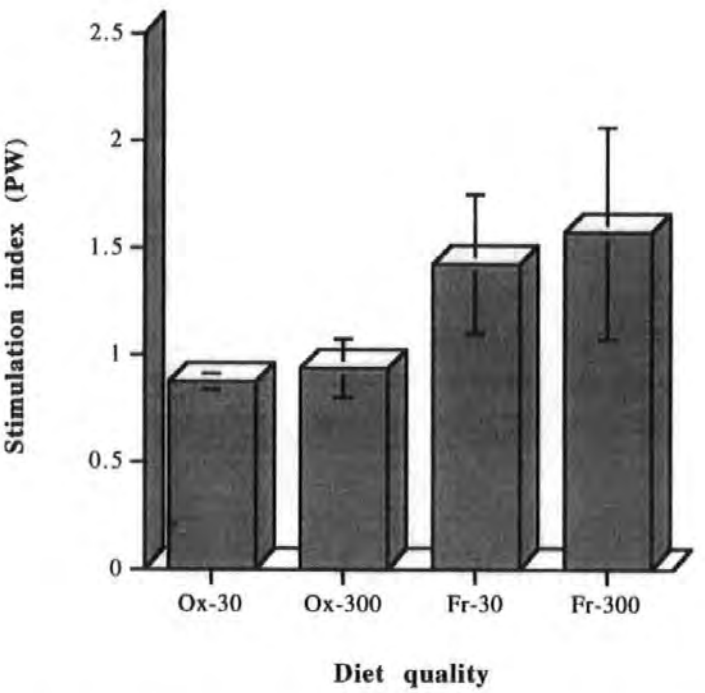


Fig. 4.9: SI of kidney lymphocytes from turbot (*S. maximus*) stimulated with Pokeweed mitogen

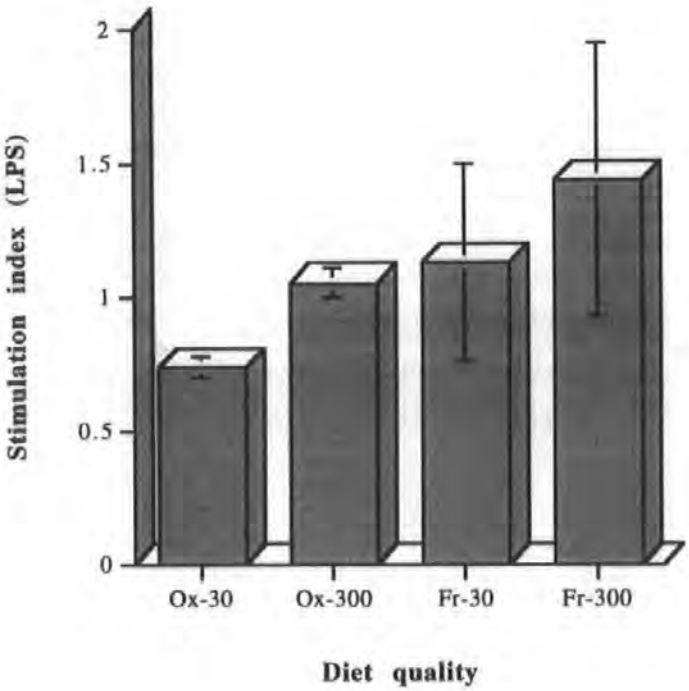


Fig. 4.10: SI of kidney lymphocytes from turbot (*S. maximus*) stimulated with lipopolysaccharide (LPS)

4.5.2 - Humoral

4.5.2.1 - Lysozyme assay

The data for serum lysozyme activity are presented in Fig. 4.11 and represent the mean of all the individuals tested ± 1 SE. Seven individuals were tested from oxidised oil and low vitamin E fed fish, 11 individuals from the oxidised oil and high vitamin E fed group, 15 from the fresh oil and low vitamin E and 18 from the fresh oil and high vitamin E fed fish. Statistical analysis (Kruskal-Wallis test) showed a significant difference in the lysozyme activity for the four treatments ($p = 0.0001$). Fish fed on oxidised oil had a lower serum lysozyme activity compare with fish fed on fresh oil.

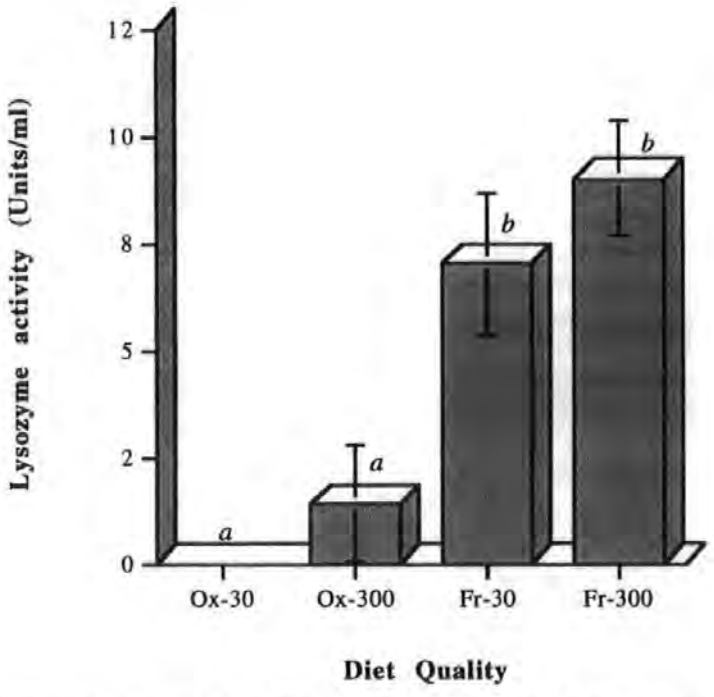


Fig. 4.11: Serum lysozyme activity of turbot (*S. maximus*) fed for 14 weeks with different levels of vitamin E and either oxidised or fresh oil. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.6.2.2 - Protein assay

The same number of individuals were tested in the protein assay determination as for the lysozyme assay. The data are shown in Fig. 4.12 and are the mean of all the individuals tested ± 1 SE. Statistical analysis using the Kruskal-Wallis test revealed a significant difference between the four dietary treatments ($p = 0.001$).

A higher protein concentration was measured in the serum of fish fed with fresh oil compared with fish fed with oxidised oil.

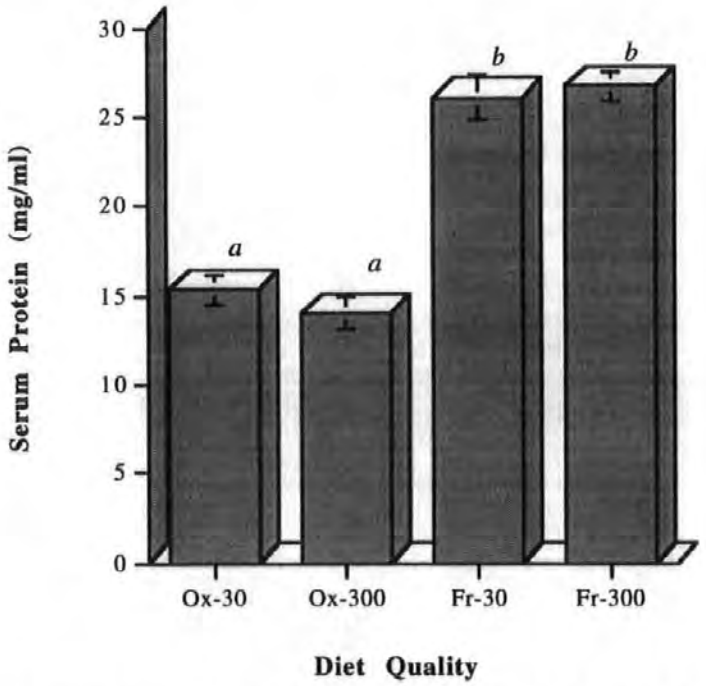


Fig. 4.12: Total serum protein levels of turbot (*S. maximus*) fed for 14 weeks with different levels of vitamin E and either oxidised or fresh oil. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

4.7 - DISCUSSION

A summary of the results from the assays showing significant differences between the dietary treatments is presented in Table 4.9.

Test/Fatty acid	Ox-30	Ox-300	Fr-30	Fr-300	Statistic
Liver vitamin E (mg/kg)	13.8 ± 3.2^{a}	18.9 ± 2.7^{ac}	3.9 ± 0.5^{b}	31.6 ± 6^{c}	$p = 0.0001$
Final length (cm)	10.7 ± 0.3^{ab}	10 ± 0.2^{a}	12 ± 0.5^{bc}	12.8 ± 0.5^{c}	$p = 0.0002$
Final weight (g)	16.8 ± 1.4^{ab}	13 ± 1^{a}	31 ± 3.8^{bc}	37 ± 3.5^{c}	$p = 0.0001$
Phagocytic cells (%)	68 ± 3.2^{a}	67 ± 4.9^{a}	29 ± 2.6^{b}	22 ± 2.6^{b}	$p<0.001$
Lymphocytes (%)	12 ± 3^{a}	11 ± 2.2^{a}	28 ± 2.3^{b}	22 ± 1.5^{b}	$p<0.001$
Thrombocytes (%)	20 ± 2.4^{a}	21 ± 3.6^{a}	43 ± 3.4^{b}	55 ± 2.2^{c}	$p<0.001$
Phagocytosis (No particles/ mg)	$4.6 \times 10^{8\ ab}$	$1.42 \times 10^{8\ a}$	$4.45 \times 10^{8\ b}$	$8.6 \times 10^{8\ ab}$	$p = 0.048$
Neutral red uptake (OD/mg)	3.9 ± 3.9^{ab}	2.5 ± 1.7^{a}	12.8 ± 2.7^{b}	19 ± 3.4^{b}	$p = 0.0033$
Lysozyme assay (Units/ml)	0 ± 0^{a}	1.4 ± 1.3^{a}	7 ± 1.7^{b}	9 ± 1.4^{b}	$p = 0.001$
Protein assay (mg/ml)	15.3 ± 0.9^{a}	14 ± 0.9^{a}	26.1 ± 1.3^{b}	26.7 ± 0.8^{b}	$p = 0.001$

Table 4.9: Summary of results. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

Vitamin E levels in diets were affected by oxidation of dietary lipids. Indeed, even though similar levels were added to Ox-30 and to Fr-30 diets, lower vitamin E levels were measured in the Ox-30 than in the Fr-30 diet after processing. A similar trend was noticed between Ox-300 and Fr-300 diets. The vitamin E included was in the form of α-tocopherol acetate, an ester form of α-tocopherol which is very stable. This molecule is hydrolysed in the gut, releasing the alcohol α-tocopherol which is then able to act as an antioxidant. Forster *et al.* (1988) who also used α-tocopherol acetate, noticed a similar decrease in vitamin E levels in diets prepared with oxidised herring oil compared with diets prepared using the same level of vitamin E and non-oxidised herring oil. It is possible that partial hydrolysis of α-tocopherol acetate occured during diet manufacture, leading to liberation of α-tocopherol which was reduced by oxidised lipids, causing a decrease in the detectable levels of vitamin E.

The liver vitamin E content measured at the end of the trial was lower in fish fed Fr-30 than in the group fed Ox-300 and Fr-300. However, liver vitamin E was also lower than in Ox-30 fed fish. Decreased tissue levels of vitamin E have been reported to result from consumption of diets prepared with oxidised oil in various fish species (Obach and Baudin-Laurencin; 1992, Obach *et al.*, 1993; Baker and Davies, 1997). Thus, the decreased vitamin E levels measured in livers of Fr-30 fed fish compared with Ox-30 fed fish was unexpected. An explanation could be that as fish fed with Fr-30 grew more than fish fed oxidised oil there was a greater requirement for the incorporation of vitamin E into tissues. This could lead to higher mobilisation of vitamin E stored in the liver in fish fed Fr-30 compared with fish fed Ox-30.

Lipid oxidation results in the formation of hydroperoxides which in turn can degrade to a variety of products including to aldehydes, ketones and alcohols (Bell and Cowey, 1985). Watanabe and Hashimoto (1968) induced production of secondary oxidation products in saury oil by blowing air into the oil to oxidise it, and showed that these products led to muscular dystrophy. Although such products were not measured in the present study, their formation would have been induced by oxidation of the oil. Gas chromatography was used to determine the total fatty acid composition of diets and livers to investigate the effect of oxidative stress on fatty acid composition.

Despite a slight decrease in dietary levels of (n-3) fatty acids in the oxidised oil diet, only the 18:3 (n-6) level was shown to differ statistically between the dietary regimes. Hung *et al.* (1983) demonstrated that diets prepared with oxidised herring oil did not show major differences in the composition of their PUFAs when the oil had been oxidised by bubbling air through it for 1348 hours despite a slight decrease in (n-3) PUFAs. In contrast, a greater loss of (n-3) PUFAs was characterised when air was blown through the oil for 1540 hours. In the present study the cod liver oil was oxidised by bubbling air through it for 700 hours which is considerably less than the time used by Hung *et al.* It is therefore not surprising that differences could not be characterised between the diets prepared with such oils.

In contrast, a decrease in the proportion of (n-3) PUFAs was generally detected in livers of fish fed oxidised oil diets and/or low vitamin E diets. This correlates with the lower levels of these PUFAs in the oxidised diets and is not surprising as PUFAs are more prone to

oxidation and degradation by oxidative stress than are saturated fatty acids. However, one of the PUFAs, 20:4 (n-6) was found at a higher level in livers of fish fed with oxidised oil compared with fish fed with fresh oil. The levels of this PUFA were not statistically different between the diets and even decreased slightly with both oxidation of the lipids and low vitamin E supplementation.

An increase of 20:4 (n-6) and 22:6 (n-3) has been reported in livers of the African catfish (*Clarias gariepinus*) fed for 8 weeks with oxidised oil diets compared with fish fed on fresh oil diets (Baker and Davies, 1996, 1997). Although a signficant difference was not characterised in the present study, the level of 22:6 (n-3) was increased in livers of fish fed on oxidised oil diets. Several hypotheses have been advanced to explain this increase (Baker and Davies, 1996, 1997) although it is difficult without further tests to affirm which mechanism underlies the changes observed in the proportions of those two PUFAs. The PUFAs 20:4 (n-6), arachidonic acid, and 22:6 (n-3), docosahexaenoic acid, are potential precursors of prostaglandins in fish (Bell *et al.*, 1986). A mechanism of regulation may exist to maintain synthesis of these two PUFAs in oxidised oil fed fish, as they can play an important role in inflammatory responses. However the mechanisms by which these two PUFAs are maintained at higher levels in the diet must differ. Indeed turbot are known to have poor elongase and Δ5 desaturase activities; thus they are unable to synthesize 20:4 (n-6) de novo and are dependent on external sources for this PUFAs. (Cowey *et al.*, 1976; Gatesoupe *et al.*, 1977a,b; Leger *et al.*, 1979; Linares and Henderson, 1991). In contrast, turbot are able to desaturate and elongate PUFAs of longer chain length (Linares and Henderson, 1991) to synthesize HUFAs. Calder (1997) describes the mechanisms of synthesis of 22:6 (n-3) from eicosapentaenoic acid in rat liver as a four step process. The last step is a β-oxidation taking place in peroxisomes. In the present study the level of 22:6 (n-3) was increased in livers of fish fed oxdised oil or low vitamin E levels. It is possible to think that these levels have been rose by oxidative stress in fish fed oxidised oil diet or low vitamin E diets.

Turbot fed on a diet containing oxidised oil and a high vitamin E level had lower final weights than turbot fed with fresh oil and either low or high vitamin E supplementation. In addition, fish fed on oxidised oil and low vitamin E levels had a lower final weight than

fish fed on fresh oil and high vitamin E levels. Vitamin E supplementation did not influence the weight of fish fed on either oxidised or fresh oil diets.

The reduced weight of fish fed on oxidised diets was probably due to the low palatability of these diets due to strong rancid aromas, and although feeding, the amount of food ingested by oxidised oil fed fish was lower than that of fresh oil fed fish. Numerous studies have already shown that a combination of rancid feed and vitamin E depletion leads to depressed growth in various species of fish (Smith, 1979; Gallet de Saint-Aurin, 1987; Forster *et al.*, 1988; Stephan, 1991; Messager *et al.*, 1992; Baker and Davies, 1996, 1997). Moreover, diets prepared with oxidised oil contained slightly less (n-3) PUFAs than fresh oil diets and (n-3) PUFAs have been reported to increase growth of turbot (Cowey, 1976; Gatesoupe *et al.*, 1977a,b). This might have enhanced the difference in weight caused by reduced food intake.

A few cases of 'melanism' (black skin on the dorsal surface) were detected in fish fed with a combination of low vitamin E supplementation and oxidised oil. Similar observations were made in the first experiment (chapter 3) when turbot were fed on vitamin E depleted diets. These signs of pathology were previously noticed in indigenous fish of the West Indies fed an oxidised diet deficient in antioxidant (Raymond, 1988) and in rainbow trout fed vitamin E depleted diets (Frischknecht *et al.*, 1994). This would suggest that melanism is a symptom appearing in cases of oxidative stress (either depletion of vitamin E or low supplementation combined with oxidation of lipids in the diet). A comparison of fish fed Fr-30 with fish fed Ox-30 suggests that low vitamin E supplementation can prevent these symptoms under non-oxidative conditions but that oxidation of dietary lipids increases the requirement for vitamin E in the diet.

Thus, vitamin E supplementation can be used to prevent the negative effects of lipid oxidation in the diet, as previously noticed by Raymond (1988).

The percentage of phagocytic cells in blood smears from turbot was significantly increased in fish fed with oxidised oil compared with fish fed on fresh oil.

It has been reported that trout and salmon infected with *Renibacterium salmoninarum* had increased numbers of monocytes and neutrophils (Bruno and Munro, 1986). Infection

could be the explanation for the increased numbers of phagocytic cells in fish fed depleted vitamin E diets in the first experiment (chapter 3). Indeed, those fish showed signs of disease in parallel with an increased number of phagocytic cells. In the present study, signs of disease were also observed in fish fed oxidised oil from week 6. Although the nature of this infection was not investigated this may be the cause of the increased number of circulating phagocytes.

While the percentage of phagocytic cells increased, the relative percentage of both thrombocytes and lymphocytes decreased in blood smears of turbot. The decrease in relative percentage of lymphocytes and thrombocytes in the blood may only reflect the effect of the increase in circulating phagocytic cells but actual number of cells should have been counted to be able to confirm this.

Statistical difference was only noticed between fish fed Ox-300 and fish fed Fr-30. Cells from fish fed on the Fr-30 diet had a lower capacity to phagocytose zymosan particles than those from fish fed on Ox-300 diet. Phagocytosis was previously shown to increase in fish fed with high levels of vitamin E compared with fish fed with lower vitamin E levels (Blazer and Wolke, 1984; N'Doye, 1993; Verlhac and Gabaudan, 1997). Furthermore, decreased respiratory burst activity was measured in turbot (Obach and Baudin Laurencin, 1992) or sea-bass (*Dicentrarchus labrax*) (Obach *et al.*, 1993) fed on low vitamin E and oxidised oil. More recently, Dushkin *et al.* (1998) demonstrated that oxygen derivatives of cholesterol, which can be produced in cases of oxidative stress, affected a range of functions of mouse macrophages and lymphocytes *in vitro*. Pretreatment of macrophages with oxysterol decreased their IL-1 like secretion and inhibited splenocyte secretion of macrophage activating factor. Inhibition of Fc-R dependant binding and phagocytosis of sheep red blood cells was also reported, and one of the oxysterols tested also reduced the zymosan-induced chemiluminescent response of peritoneal macrophages. Two mechanisms were proposed to explain these changes. One is the regulation of 3 hydroxy-3methylglutaryl-CoA (HMG-CoA) reductase and LDL receptor; intracellular regulatory factors are believed to be responsible for this mechanism and the expression of genes other than HMG-CoA reductase appear to be modulated by these factors. The second mechanism involves a substantial modification of membrane dynamic properties which

consequently triggers several biological effects associated with membrane-linked receptors and enzymes. Inhibition of reactive oxygen intermediate species generation in peritoneal macrophages could be explained by the alteration of membrane linked NADPH-oxidase. Therefore, data from the literature favour the idea that oxidation of lipids or oxygen derivatives of lipids down-regulate phagocytosis. However, data obtained in the present study does not support this idea. The result obtained in the present study may reflect an activation of macrophages in fish fed with oxidised oil diets which gives them an enhanced ability to phagocytose zymosan particles compared with phagocytic cells from fresh oil fed fish. This does not imply that an effect of oxidation of lipids on phagocytosis such as the ones described previously does not occur, but that it might be obscured by this enhanced phagocytotic ability.

Data obtained from the neutral red uptake assay in the present study do not corroborate the results of the phagocytosis assay. Turbot fed with oxidised oil and high vitamin E showed a reduced uptake of neutral red compared with turbot fed on fresh oil, but no significant differences were characterised between turbot fed Ox-30 and turbot fed with fresh oil diets. Within the fresh or oxidised oil treatments, vitamin E did not influence the uptake of neutral red so contrary to the oxidation of lipids vitamin E may not play a determinant role.

Thus it seems that lipid oxidation affects the ability of macrophages to pinocytose neutral red. It is difficult to explain why in one case macrophages would be "stimulated" by oxidised oil to phagocytose zymosan particles but have a reduced potential to uptake neutral red. Even though phagocytosis and pinocytosis are two related processes, differences exist. Phagocytosis is the uptake of large particles into vacuoles by mechanisms that are clathrin independent and usually require actin polymerisation, whereas pinocytosis is usually clathrin dependent and generally does not require actin polymerisation (Rabinovitch, 1995). Lipid oxidation of the diet may affect some aspects of the cellular machinery which are involved in pinocytosis but not in phagocytosis. Unfortunately an investigation of these processes was outside the scope of this study.

The lysozyme activity was decreased in oxidised oil fed fish compared with fresh oil fed fish. Similar results were previously obtained with sea-bass or turbot fed with oxidised oil

diets (Obach *et al.*, 1993). Lysozyme activity has been demonstrated in various fish species to be associated with leucocyte rich tissues and it has been found at sites where it could play a role in defence against pathogens (Fletcher and White, 1973; Fänge *et al.*, 1976; Murray and Fletcher, 1976; Grinde *et al.*, 1988; Lindsay, 1988). Granulocytes and macrophages appear to be the principal secretory cells of this enzyme. It has been suggested that the lysozyme level in blood could be used to diagnose the disease condition in fish, as disease influences the number of circulating leucocytes and consequently the concentration of lysozyme (Fletcher and White, 1973). However, although the number of circulating leucocytes was not measured, the different categories of white blood cells showed an increase in phagocytic cells for fish fed on oxidised oil diets. Thus it seems unlikely that this could account for the variation in lysozyme activity observed in plasma.
In his study Obach (1993) advanced a theory to explain this decreased lysozyme activity. Indeed, lipid peroxides have been shown to affect the enzymatic activity in higher vertebrates (Kaneda and Miyazawa, 1987). Kanazawa *et al.* (1975) demonstrated an inactivation of lysozyme *in vitro* by secondary degradation products produced from autoxidation of linoleic acid. This mechanism might account for the decrease of lysozyme activity in the plasma of turbot fed an oxidised oil diet in the present study.
The reduction in lysozyme activity may explain the greater succeptibility to infection and the increased mortality of fish on oxidative oil treatments.

There was a net decrease in total serum protein concentration in fish fed on oxidised oil compared with fish fed on fresh oil after 14 weeks. The data available in the literature concerning total serum protein varies with the species studied (Yamashita, 1967; Blazer and Wolke, 1984; Evenberg *et al.*, 1986; Hardie *et al.*, 1990; Messager *et al.*, 1992). In the present study, fish showing lower protein values were among the group which had shown disease. Evenberg *et al.* (1986) suggested three potential mechanisms to explain the decreased protein levels: (1) loss of protein through vascular leaking, (2) impaired synthesis of protein resulting from liver damage and anorexia in diseased fish, (3) (non-) specific proteolysis of serum protein. The third option is from the observation of Duswald (1985) who reported that sepsis of gram-negative bacteria in mammals can be accompanied by non-specific degradation of almost all serum proteins. Liver lipoid

degeneration has been observed in rainbow trout fed with rancid diets and depleted in vitamin E and C (Smith, 1979), and signs of liver ceroidosis and necrosis were also detected in trout fed with highly or extremely oxidised oil without vitamin E supplementation (Moccia *et al.*, 1984). Liver lesions were not observed in sea-bass fed for 23 weeks with oxidised oil and antioxidant deficiency (Messager *et al.*, 1992). However, preliminary observations on the same species showed liver degeneration in fish farmed in tropical environments and fed rancid diets, deficient in vitamin E. Therefore, impaired protein synthesis resulting from liver damage may underlie the changes observed between groups of fish in the present work.

CHAPTER 5 - EFFECT OF VITAMIN E AND PUFAS ON THE IMMUNE SYSTEM OF TURBOT (*SCOPHTHALMUS MAXIMUS*)

5.1 - INTRODUCTION

Epidemiological evidence correlates a high dietary intake of fish oils in Greenland Eskimo populations with low death rates from breast cancer (Bang *et al.*, 1976) and a lowered incidence of inflammatory and autoimmune diseases (Kromann and Green, 1980) and ischaemic heart diseases (Bang and Dyerberg, 1980). Following these observations numerous studies focused on the effect of lipids on the immune system of mammals. In general animals fed higher levels of PUFAs have reduced immunocompetence and PUFAs tend to be seen as immunodepressors. However, different qualities of PUFAs can exert different effects on immune competence as suggested by Bang and Dyerberg (1980) in their investigations in Greenland Eskimos. Indeed, the low incidence of ischemic heart diseases could be correlated more with the variety of PUFAs found in the diet rather than the total amount of PUFAs. The higher proportion of (n-3) PUFAs in the diet compared to their (n-6) counterpart seemed to be responsible for the changes observed. More recently, studies revealed that indeed dietary fish oils, via their (n-3) fatty acid components, can help autoimmune and inflammatory conditions by suppressing some immune cells (Calder and Newsholme, 1993).

Several authors have shown a relationship exists between the requirements of animals for α-tocopherol and the lipid contents of the diets (Watanabe *et al.*, 1977, 1981a,b; Roem *et al.*, 1990; Peck, 1994). An increased level of dietary lipid or an increased level of unsaturation of dietary lipid enhances the requirement for dietary vitamin E in various species.

Moreover, vitamin E is necessary in the diet of different fish to sustain a fully functioning immune system (Blazer and Wolke, 1984; Hardie *et al.*, 1990; Blazer and Wolke, 1991; Hardie *et al.*, 1991; Verlhac *et al.*, 1991; Furones *et al.*, 1992; Wise *et al.*, 1993; Verlhac and Gabaudan, 1997) and some studies indicate a positive effect of enhanced levels of vitamin E on immune competence. Therefore, adding vitamin E could counterbalance or enhance the immunomodulatory role of different PUFAs .

Turbot, have been the subject of numerous studies concerning fatty acid requirements and the influence of dietary PUFAs on growth and fatty acid composition of different organs (Cowey *et al.*, 1976 a,b; Gatesoupe *et al.*, 1977a,b; Léger *et al.*, 1979; Bell *et al.*, 1985a,b,

1995a,b,c; Tocher and Sargent, 1987; Linares and Henderson, 1991; Castell *et al.*, 1994). Turbot have poor ability for elongation and desaturation of (n-3) and (n-6) PUFAs of short chain length to their homologous high polyunsaturated fatty acids (HUFAs), 20:4 (n-6), 20:5 (n-3) and 22:6 (n-3). Therefore, the fish are dependent on external sources of HUFAs and require a correct balance of these three HUFAs in their diets.

In a series of recent studies Bell *et al.* (1995a,c) showed a relationship between dietary lipids and PG production by different organs of juvenile turbot. This reveals the importance of considering dietary lipids not only for their direct structural role in the plasma membrane but also for their role as eicosanoid precursors and thus in inflammatory processes.

Despite the abundance of data on the influence of lipid nutrition on growth and fatty acid composition of different tissues of turbot, only one investigation, looking at the influence of such dietary treatments on the immune response, has been carried out (Obach, 1993). Since then different studies have yielded more information on the effect of dietary lipids on PG production and on lipid composition of different organs of turbot (Bell *et al.*, 1995a,c).

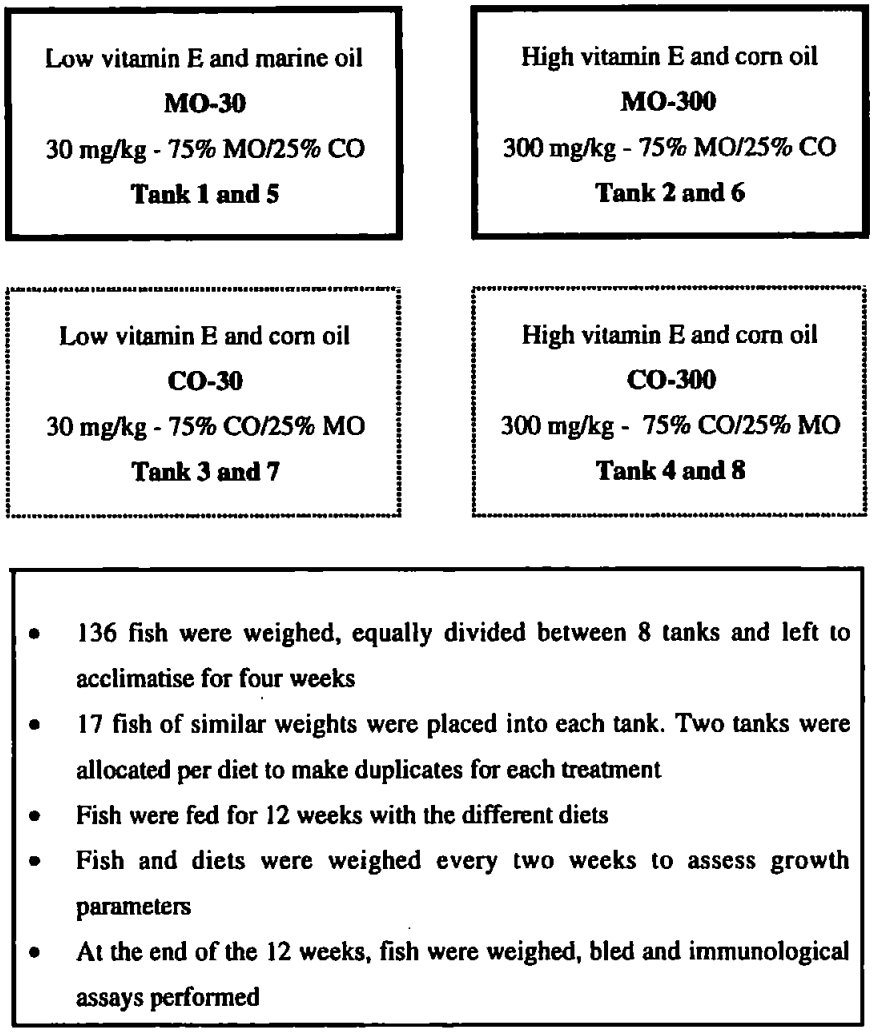
The following study was undertaken to investigate the effect of different levels of vitamin E and different oil qualities upon a range of specific and non-specific defence mechanisms in juvenile turbot.

Two different oils were used for this experiment, cod liver oil (rich in (n-3) PUFA) and corn oil (richer in (n-6) PUFA). Combinations of various proportions of these two oils with two different levels of vitamin E were used to prepare four different diets as follows: 1), high percentage of marine oil and 30 mg of vitamin E/kg of diet, 2) high percentage of marine oil and 300 mg of vitamin E/kg of diet, 3) high percentage of corn oil and 30 mg of vitamin E/kg of diet, 4) high percentage of corn oil and 300 mg of vitamin E/kg of diet. This should enable a determination of whether oil quality affects the immune response of turbot in a similar way to growth parameters or whether the beneficial effects on growth can be detrimental to some immune parameters.

The supplementation of each diet with different levels of vitamin E should allow an investigation of the immunomodulatory role of α-tocopherol with different lipid sources.

5.2 - EXPERIMENTAL PROTOCOL

The following figure represents the experimental protocol:



5.3 - COMPOSITION OF THE EXPERIMENTAL DIET

5.3.1 - Chemical composition of the diets

The ash, moisture, protein and lipid composition of the diets were determined and the results are presented in Table 5.1. On average the diets were composed of 3.7% moisture, 11.2% ash, 51% protein and 14.8% lipids. All the figures show constancy amongst the different diets with the exception of lipids for which an unexpected drop was detected for the CO-300 diet (10.6%) compared to the other diets (around 16%). This difference was

unexpected as all the diets were formulated using the same quantity of oil although differing in quality.

Diet	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
MO-30	4.2	11.3	51.1	15.5
MO-300	3.8	11	51.2	16.1
CO-30	3.8	11.2	51	17
CO-300	3.1	11.3	51.3	10.6

Table 5.1: Composition of the test diets expressed as a percentage of wet weight

5.3.2 - Analysis of vitamin E levels

Dietary vitamin E levels were measured by HPLC (Roche, St Louis, France). The data are presented in Table 5.2 as the mean of two replicates ± 1 S.E. A Kruskal-Wallis test did not show any significant differences in vitamin E level between the different diets.

Diet	N	Included (mg/kg)	Measured (mg/kg)
MO-30	2	30	127 ± 10
MO-300	2	300	258 ± 127
CO-30	2	30	38.5 ± 18.5
CO-300	2	300	137.5 ± 30.5

Table 5.2: Levels of vitamin E included in the diets and measured after manufacturing

5.3.3 - Lipid analysis

The lipid composition of diets are presented in Table 5.3 as the mean of 3 samples ± 1 S.E.. Significant differences between diets were characterised for some of the PUFAs and the p values are listed in the right hand column (Kruskal-Wallis, $p < 0.05$). Although an

overall difference was detected using the Kruskal-Wallis test, pairwise comparisons using the Dunn-Sidak method (Ury, 1976), failed to show any differences between individual diets. This is probably caused by the small number of sample used.

Diets formulated with 75% marine oil had a higher proportion of (n-3) polyunsaturated fatty acids (PUFAs) compared with diets formulated with a high percentage of corn oil which had a higher proportion of (n-6) PUFAs. The (n-3)/(n-6) PUFAs ratio was around 2.5 for diets formulated with 75% marine oil compared with 0.6 for diets formulated with 75% corn oil. Amongst the (n-3) PUFAs, 20:5 (n-3) and 22:6 (n-3) were responsible for most of the difference observed in the total (n-3) PUFAs between the MO and CO diets. Amongst (n-6) PUFAs, 18:2 (n-6) appears to be the fatty acids contributing most to the differences in figures for total (n-6) PUFAs between MO and CO diets.

Diets formulated with marine oil had an increased percentage of saturated fatty acids and a decreased percentage of PUFAs compared with diets formulated with corn oil.

Fatty Acid	MO-30	MO-300	CO-30	CO-300	STATS
14: 0	5.4 ±0.1	5.3 ±0.09	3.2 ±0.03	3.1 ±0.02	*p = 0.023*
15: 0	0.4 ±0.01	0.4 ±0.006	0.2 ±0.003	0.2 ±0	*p = 0.028*
16: 0	16.6 ±0.3	16.7 ±0.2	14.4 ±0.3	14.1 ±0.1	*p = 0.036*
16: 1 (n-7)	7.1 ±0.1	6.9 ±0.09	4.3 ±0.04	4.2 ±0.05	*p = 0.026*
16: 2 (n-3)	0.6 ±0.01	0.6 ±0.008	0.3 ±0.003	0.29 ±0.003	*p = 0.017*
16: 3 (n-3)	1.1 ±0.4	0.6 ±0.07	0.2 ±0.006	0.3 ±0.005	*p = 0.031*
16: 4 (n-3)	1.1 ±0.01	1 ±0.01	0.7 ±0.2	0.4 ±0.01	
18: 0	3.7 ±0.07	3.7 ±0.1	3.3 ±0.2	3.3 ±0.01	
18: 1 (n-9)	15.7 ±0.2	15.4 ±0.4	20.7 ±0.2	20.3 ±0.01	*p = 0.025*
18: 1 (n-7)	3.4 ±0.04	3.5 ±0.08	2.6 ±0.1	2.5 ±0.04	*p = 0.038*
18: 2 (n-6)	9.9 ±0.1	10.2 ±0.6	26.1 ±0.1	26.8 ±0.1	*p = 0.025*
18: 3 (n-6)	0.2 ±0.003	1.3 ±1.1	0.1 ±0.05	0.04 ±0.04	*p = 0.034*
18: 3 (n-3)	0.8 ±0.01	1 ±0.3	0.8 ±0.03	0.9 ±0.005	
18: 4 (n-3)	1.9 ±0.06	1.8 ±0.03	1.2 ±0.01	1.2 ±0.01	*p = 0.024*
20: 0	0.2 ±0.006	0.2 ±0.01	0.3 ±0.005	0.3 ±0	*p = 0.027*
20: 1 (n-9)	4.6 ±0.08	4.6 ±0.2	4.1 ±0.02	4.1 ±0.01	*p = 0.03*
20: 4 (n-6)	0.6 ±0.01	0.5 ±0.005	0.3 ±0.003	0.3 ±0.006	*p = 0.027*
20: 4 (n-3)	0.5 ±0.01	0.4 ±0.01	0.3 ±0.02	0.3 ±0.003	*p = 0.028*
20: 5 (n-3)	10.5 ±0.3	9.9 ±0.1	5.6 ±0.09	5.8 ±0.08	*p = 0.023*
22: 1 (n-11)	4.5 ±0.1	4.6 ±0.2	4.1 ±0.03	4.1 ±0.02	*p = 0.034*
22: 5 (n-3)	1.2 ±0.04	1.1 ±0.03	0.6 ±0.01	0.6 ±0.005	*p = 0.029*
22: 6 (n-3)	9.5 ±0.4	9.1 ±0.2	6 ±0.1	6.3 ±0.09	*p = 0.029*
24: 1 (n-9)	0.6 ±0.03	0.8 ±0.2	0.5 ±0.04	0.5 ±0.03	*p = 0.039*
Sats	26.4 ±0.5	26.4 ±0.4	21.5 ±0.4	21 ±0.1	*p = 0.039*
Monos	35.8 ±0.4	35.9 ±1	36.3 ±0.2	35.7 ±0.03	
PUFAs	37.8 ±0.9	37.7 ±1.4	42.2 ±0.2	43.3 ±0.1	*p = 0.025*
Total (n-3)	27.1 ±0.8	25.6 ±0.6	15.7 ±0.06	16.1 ±0.2	*p = 0.02*
Total (n-6)	10.7 ±0.2	12.1 ±1.7	26.5 ±0.2	27.1 ±0.1	*p = 0.024*
(n-3)/(n-6)	2.5 ±0.06	2.2 ±0.3	0.6 ±0	0.6 ±0	*p = 0.019*

Table 5.3: Fatty acid composition of diets after manufacture. PUFAs for which significant differences were detected are highlighted and the corresponding p values are presented in the right column.

5.4 - UPTAKE OF NUTRIENT

5.4.1 - Liver vitamin E

Vitamin E levels in the liver were measured by HPLC (Roche, St Louis, France). The results are presented in Fig. 5.1 as the mean of 10 individuals ± 1 S.E. A good correlation was found between the levels of vitamin E measured in the diet and the levels measured in the livers of turbot after 12 weeks of feeding ($r = 0.791$, $p < 0.01$). A Kruskal-Wallis test revealed an overall significant difference between the dietary treatments ($p = 0.0001$).

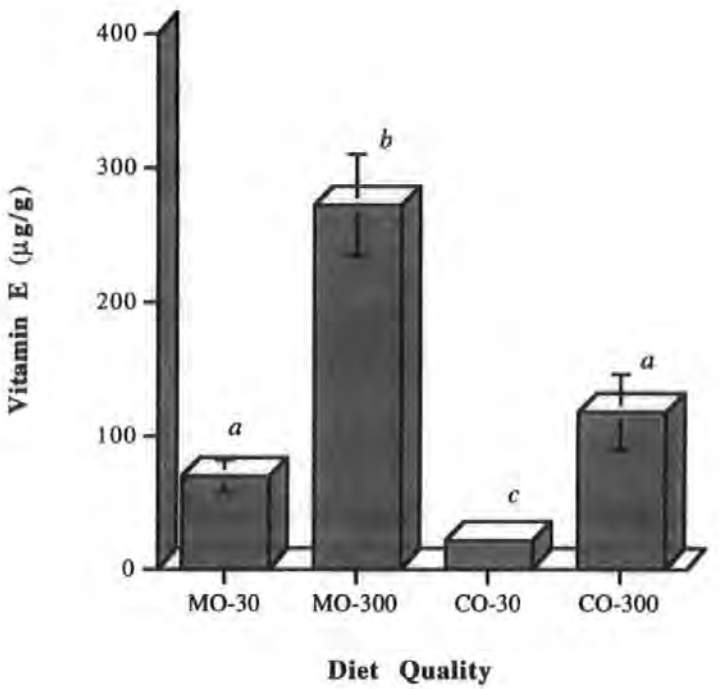


Fig. 5.1: Vitamin E levels in livers of turbot (*S. maximus*) fed for 12 weeks with different vitamin E levels and different oil qualities. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different.

Fish fed with the high percentage of corn oil showed lower vitamin E levels, about 70% lower than for the low vitamin E diet ($p = 0.0012$), and 55% lower than for the high vitamin E diet ($p = 0.0055$) compared with fish fed on high levels of marine oil and (theoretically) equivalent vitamin E levels. This parallels the differences in vitamin E levels already detected in the diets after manufacturing.

5.4.2 - Fatty acid composition of the liver

Lipid analysis of the liver was carried out after the twelve week feeding period by GC and the data are presented in Table 5.4 as the mean of three individuals tested ± 1 SE.
A Kruskal-Wallis non-parametric test was used to test the differences between dietary treatments and the p values are presented in the right hand column when significant differences were characterised. Although an overall difference was detected by Kruskal-Wallis test, a pairwise comparison using the Dunn-Sidak method (Ury, 1976) failed to show any differences between individual diets. This is probably caused by the small number of sample used.

Livers of fish fed with the marine oil diet showed increased levels of saturated fatty acids and total (n-3) PUFAs compared with livers of fish fed with corn oil diets. In contrast fish fed with the corn oil diet had increased proportions of total (n-6) PUFAs compared to fish fed with marine oil. Amongst the (n-6) PUFAs, 18:2 (n-6) seemed to be responsible for most of the differences observed between the dietary treatments as the percentage rose from 11% in marine oil fed fish to 31% in corn oil fed fish. Amongst the (n-3) PUFAs, the most important variations were observed for 20:5 (n-3), 22:5 (n-3) and 22:6 (n-3) for which percentage composition of livers in corn oil fed fish seemed to drop to a half or a third of the value found in marine oil fed fish.
Finally it is important to note that the ratio of (n-3) PUFAs to (n-6) PUFAs in the livers of fish reflected the ratio given in the diet with a value around 2.8 for fish fed with mainly marine oil and dropping to about 0.5 for fish fed mainly with corn oil.

Fatty Acid	MO-30	MO-300	CO-30	CO-300	STATS
14: 0	5.4 ±0.9	5.7 ±0.2	4.1 ±0.3	3.6 ±0.2	
15: 0	0.5 ±0.03	0.5 ±0.008	0.3 ±0.01	0.3 ±0.006	*p = 0.027*
16: 0	17.9 ±1	17.2 ±0.4	15.5 ±0.3	15.3 ±0.7	
16: 1 (n-7)	6.5 ±1.4	6.9 ±0.4	5 ±0.09	4.6 ±0.3	
16: 2 (n-3)	0.8 ±0.1	0.5 ±0.03	0.3 ±0.04	0.1 ±0.07	*p = 0.022*
16: 3 (n-3)	0.5 ±0.05	0.5 ±0.08	0.4 ±0.07	0.1 ±0.06	
16: 4 (n-3)	0.4 ±0.2	0.5 ±0.03	0.2 ±0.03	0.1 ±0.07	
18: 0	3.4 ±1.2	2.6 ±0.3	1.7 ±0.04	2.2 ±0.4	
18: 1 (n-9)	12.7 ±2.2	13.2 ±0.4	18.4 ±0.1	18 ±0.5	*p = 0.036*
18: 1 (n-7)	3.7 ±0.3	3.7 ±0.01	2.9 ±0.01	2.8 ±0.04	*p = 0.023*
18: 2 (n-6)	10.6 ±2	11.7 ±0.5	31.9 ±0.7	31.4 ±1.1	*p = 0.04*
18: 3 (n-6)	0.3 ±0.006	0.3 ±0.01	0.1 ±0.008	0.1 ±0.06	*p = 0.033*
18: 3 (n-3)	0.5 ±0.1	0.6 ±0.04	0.8 ±0.01	0.8 ±0.09	
18: 4 (n-3)	1.2 ±0.4	1.3 ±0.07	1 ±0.1	0.9 ±0.1	
20: 0	0.04 ±0.04	0.1 ±0.06	0.04 ±0.04	0 ±0	
20: 1 (n-9)	2.2 ±0.5	3 ±0.2	2.6 ±0.1	2.4 ±0.05	
20: 4 (n-6)	1.4 ±0.6	1.2 ±0.3	0.4 ±0.04	0.5 ±0.1	*p = 0.04*
20: 4 (n-3)	0.6 ±0.08	0.7 ±0.04	0.4 ±0.01	0.4 ±0.02	*p = 0.036*
20: 5 (n-3)	9.5 ±0.2	9.5 ±0.4	4.7 ±0.5	5.1 ±0.1	*p = 0.038*
22: 1 (n-11)	1.1 ±0.3	1.6 ±0.2	1.3 ±0.1	1.2 ±0.09	
22: 5 (n-3)	2.5 ±0.2	2.4 ±0.2	1 ±0.07	1.1 ±0.09	*p = 0.033*
22: 6 (n-3)	18 ±5.6	15.8 ±1.1	6.5 ±0.6	8.5 ±1.3	*p = 0.028*
24: 1 (n-9)	0.3 ±0.2	0.6 ±0.03	0.4 ±0.04	0.3 ±0.02	
Sats	27.1 ±1.3	26.1 ±0.7	21.7 ±0.2	21.5 ±1	*p = 0.036*
Monos	26.6 ±4.7	28.9 ±1.2	30.5 ±0.4	29.3 ±0.8	
PUFAs	46.3 ±3.5	45 ±0.5	47.7 ±0.5	49.2 ±0.3	
Total (n-3)	34 ±4.9	31.8 ±0.7	15.2 ±1	17.3 ±1	*p = 0.033*
Total (n-6)	12.3 ±1.4	13.2 ±0.3	32.5 ±0.7	32 ±1.1	*p = 0.039*
(n-3)/(n-6)	3 ±0.8	2.4 ±0.1	0.5 ±0.03	0.5 ±0.03	*p = 0.029*

Table 5.4: Fatty acid composition of turbot (*S. maximus*) livers. PUFAs for which significant differences were detected are highlighted and the corresponding p values are presented in the right column

5.5 - GROWTH PERFORMANCE

5.5.1 - Growth

Fig. 5.2 shows the growth of turbot during the 12 weeks of the feeding experiment presented as the increase in average weights (mean of N individuals) every two weeks of the experimental trial.

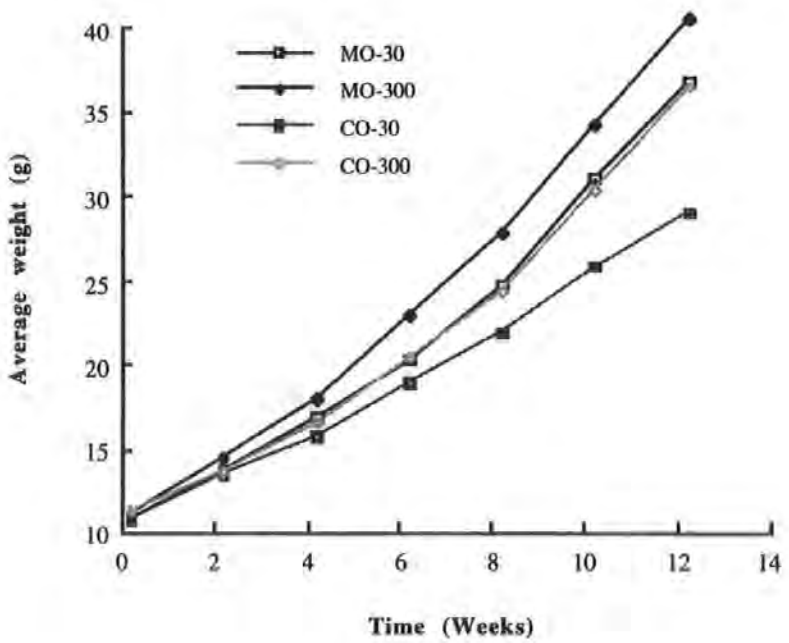


Fig. 5.2: Weight recordings from turbot (*S. maximus*) fed for 12 weeks with various levels of vitamin E and different oil qualities

Table 5.5 presents the final weights and lengths of turbot, data presented are the mean of N individuals ± 1 SE. A Kruskal-Wallis test showed a significant difference between the four dietary treatments for both final length, ($p = 0.022$) and final weights ($p = 0.0077$). Fish fed with high percentages of corn oil and low vitamin E levels showed the lowest final average weights, whereas fish fed high vitamin E levels and 75% marine oil showed the highest final weights.

Diet	N	Weight (g)	Length (cm)
MO-30	33	31.9 ± 2.6^{ab}	12 ± 0.3^{ab}
MO-300	36	35.8 ± 2.8^{a}	12.4 ± 0.3^{a}
CO-30	35	24.7 ± 1.8^{b}	11.2 ± 0.2^{b}
CO-300	32	31.0 ± 2.6^{ab}	11.9 ± 0.3^{ab}

Table 5.5: Weight and length of turbot (*S. maximus*) after the 12 week feeding period. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

5.5.2 - Specific Growth Rate (SGR)

Table 5.6. presents the cumulative SGR and FCR as the mean of 2 replicates ± 1 SE, N represents the number of fish per replicates.

Statistical analysis did not show any significant differences between dietary treatments (Kruskal-Wallis, $p < 0.05$).

Diet	N	SGR	FCR
MO-30	16	1.5 ± 0.08	1.0 ± 0.02
MO-300	16	1.6 ± 0.06	1.0 ± 0.03
CO-30	16	1.2 ± 0.03	1.2 ± 0.01
CO-300	16	1.4 ± 0.08	1.1 ± 0.03

Table 5.6: Cumulative SGR and FCR of turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E and different oil qualities

5.5.3 - Feed Conversion Ratio (FCR)

Data for cumulative FCR are presented in Table 5.6 as the average of 2 replicates ± 1 S.E. (N fish per replicate). Statistical analysis using the Kruskal-Wallis test did not show any significant differences between dietary treatments for $p < 0.05$.

5.5.4 - Behavioural recordings

At the start of the trial, all fish were feeding well and did not show any differences in appetite between the different tanks. However, from week 4 a slight increase in the quantity of diet eaten by the fish fed with marine oil and high vitamin E was noticed compared with fish fed on the same oil quality and lower levels of vitamin E. The same pattern was noticed within the group of fish fed on high percentages of corn oil. Fish fed with corn oil also showed a loss in appetite compared with fish fed on marine oil. However, this could be correlated with differences in the size of the fish which influenced dietary requirements, with bigger fish requiring more food. Indeed, a calculation of food intake per percent body weight revealed that although fish were fed *ad libitum*, the quantity of food they were ingesting as a percentage of body weight was remarkably constant between the four dietary treatments.

At week 9 one fish fed on diet 3 (low level of vitamin E and corn oil) showed signs of melanism. On the two following weeks two other fish in the same tank (tank 7), showed the same signs but this phenomenon did not appear in the duplicate tank. No other signs of melanism were detected in any of the other treatments.

No cases of mortality occurred during the experiment that could be related to the nature of the diet.

5.6 - ASSAYS FOR IMMUNOCOMPETENCE

5.6.1 - Cellular

5.6.1.1 - Haematocrit

Haematocrit, leucocrit and plasma percentages of turbot are presented in Table 5.7 as the mean of N individuals ± 1 SE. A Kruskal-Wallis test did not show any differences between the treatments ($p < 0.05$).

Diet	N	Haematocrit (%)	Leucocrit (%)	Plasma (%)
MO-30	31	17.6 ± 0.9	1.0 ± 0.08	81.4 ± 0.8
MO-300	27	17.0 ± 0.7	1.0 ± 0.07	82.0 ± 0.7
CO-30	28	16.8 ± 0.6	0.8 ± 0.07	82.4 ± 0.6
CO-300	26	18.1 ± 0.8	0.9 ± 0.09	81.0 ± 0.8

Table 5.7: Haematocrit, leucocrit and plasma percentages of turbot (*S. maximus*) after the 12 week feeding period

5.6.1.2 - Blood smears

Percentages of phagocytes, thrombocytes and lymphocytes are presented in Table 5.8 as the mean of N individuals ± 1 SE. A Kruskal-Wallis test failed to detect any significant differences in the number of phagocytes between the four dietary treatments. However, both thrombocytes ($p = 0.0165$) and lymphocytes ($p = 0.0161$) were found in significantly different proportions in fish fed with different diets. The proportion of thrombocytes was increased in the CO-300 fed fish compared with the marine oil fed fish, whereas the proportion of lymphocytes was increased in MO-300 fed fish compared with MO-30 and CO-300 fed fish.

Diet	N	.Phagocytes (%)	Lymphocytes (%)	Thrombocytes (%)
MO-30	33	21 ± 3	37.2 ± 2.7 ^{a}	41.8 ± 2.2 ^{a}
MO-300	36	11.7 ± 1.5	48.4 ± 2.4 ^{b}	39.9 ± 2.2 ^{a}
CO-30	35	14.7 ± 2	42.9 ± 3.5 ^{ab}	42.1 ± 2.3 ^{ab}
CO-300	32	11 ± 1.2	37.4 ± 3.4 ^{a}	51.2 ± 3 ^{b}

Table 5.8: Differential leucocyte counts of turbot (*S. maximus*) blood smears after the 12 week feeding period. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

5.613 - Phagocytosis

The data for phagocytosis are presented in Fig. 5.3 as the number of particles ingested per mg of protein and are the mean of N individuals ± 1 SE. Seven individuals were tested for the MO-30 diet, 6 individuals for the MO-300 and CO-30, and 8 individuals for the CO-300 diet. A Kruskal-Wallis test did not detect any significant differences between the 4 treatments ($p < 0.05$).

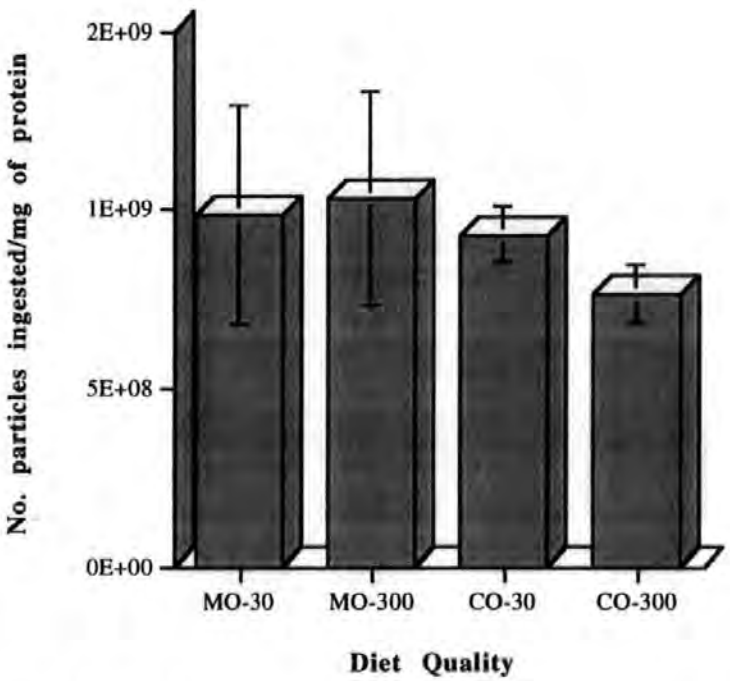


Fig. 5.3: Phagocytosis by kidney leucocytes taken from turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E and different oil qualities

5.6.1.4 - Uptake of neutral red.

The data for uptake of neutral red are presented in Fig 5.4 as the mean of 6 individuals ± 1 SE. Statistical analysis did not characterise any differences between the four dietary treatments for (Kruskal-Wallis, $p < 0.05$).

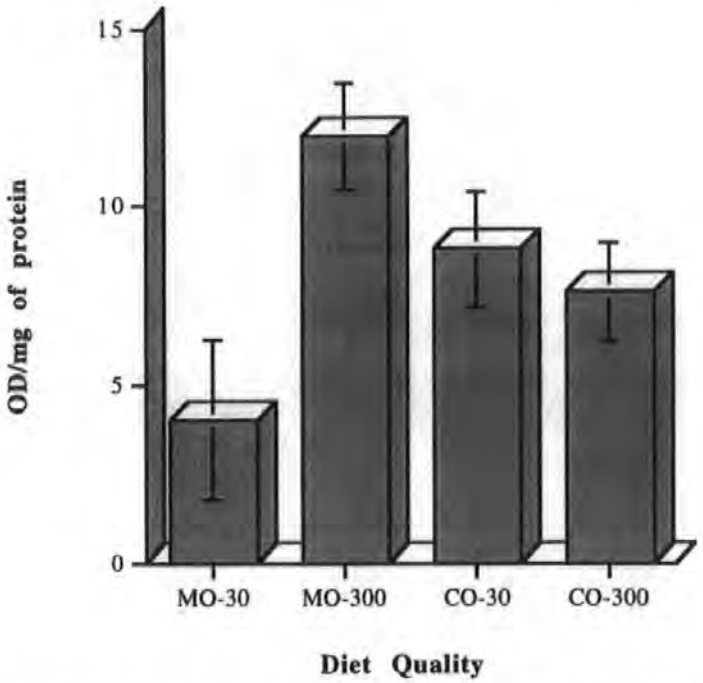


Fig. 5.4: Uptake of neutral red by kidney leucocytes from turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E and different oil qualities

5.6.1.5 - Cell proliferation assay

The results, expressed as a stimulation index (SI) are the mean of N individuals ± 1 SE. Eleven individuals were tested for MO-30 and MO-300, 13 for CO-30 and 12 for CO-300 fed fish. The different mitogens used for stimulation of proliferation were Concanavalin A (data presented in Fig. 5.5), lipopolysaccharide (data presented in Fig. 5.6) and pokeweed mitogen (data presented in Fig. 5.7).

No significant differences could be demonstrated in the proliferation of lymphocytes when stimulated with Con A or PW mitogen. (Kruskal-Wallis test, $p < 0.05$).

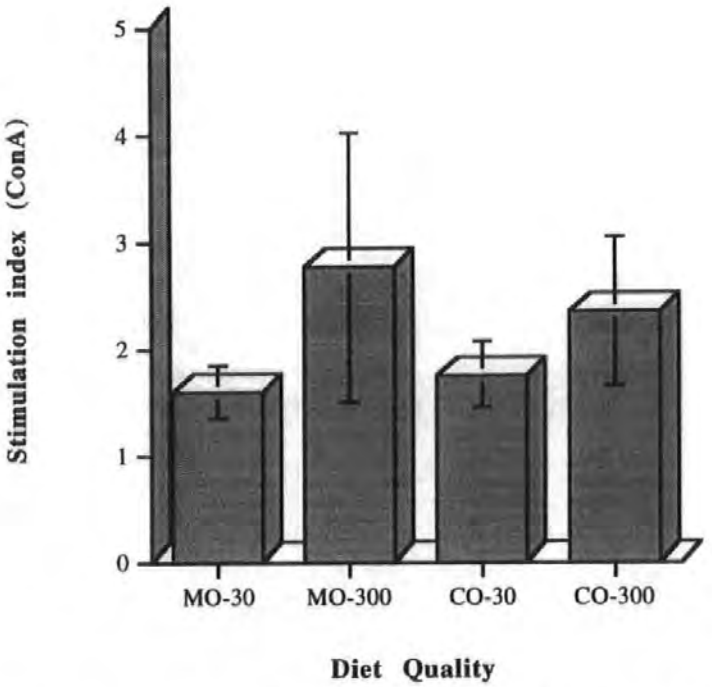


Fig. 5.5: Cell proliferation assay of kidney lymphocytes from turbot (*S. maximus*) stimulated with Concanavalin A

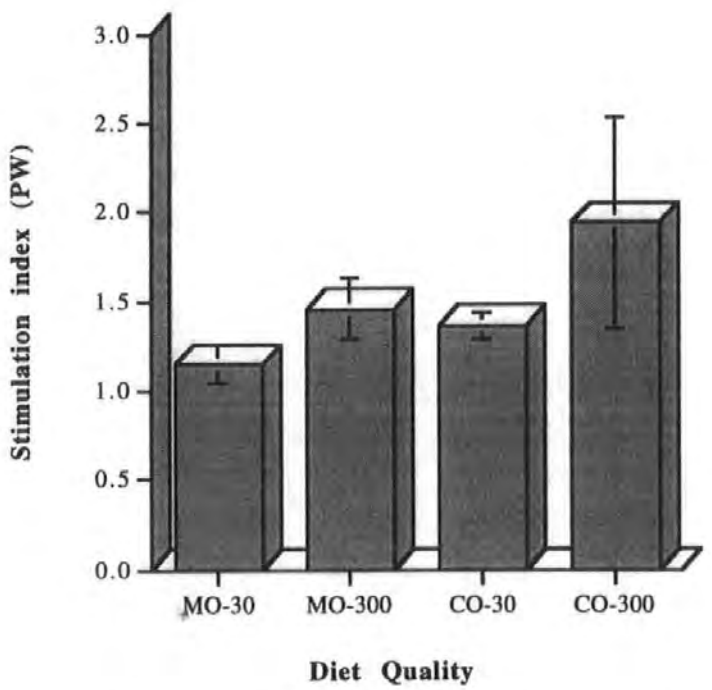


Fig. 5.6: Cell proliferation assay of kidney lymphocytes, pokeweed mitogen stimulation

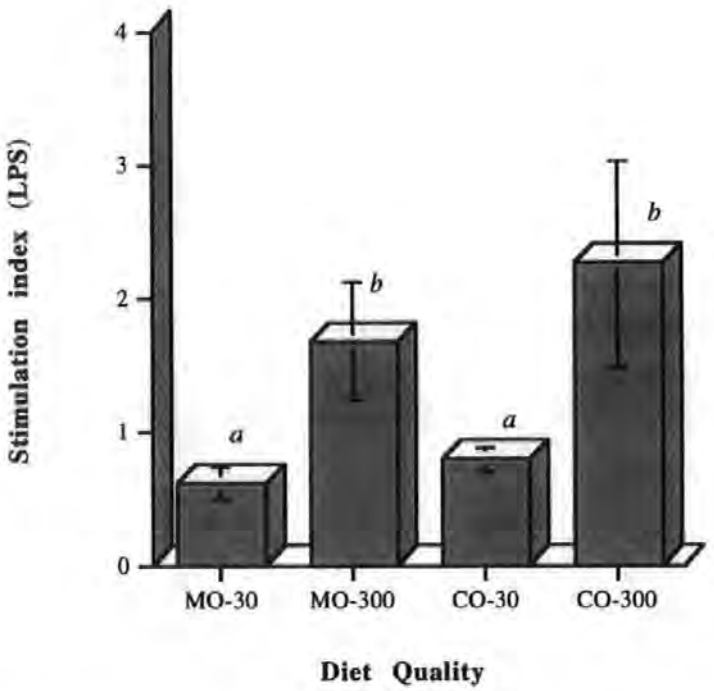


Fig. 5.7: Cell proliferation assay of kidney lymphocytes, lipopolysaccharide stimulation. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

In contrast, when using the B cell mitogen lipopolysaccharide (Fig. 5.7), a different pattern in kidney leucocyte proliferation was characterised. A Kruskal-Wallis test showed a significant difference between the four dietary treatments ($p = 0.0001$) and pairwise comparisons demonstrated an effect of vitamin E on the proliferation of kidney lymphocytes. Cell proliferation was enhanced when high vitamin E levels were included in the diets compared to treatments where low vitamin E levels were used to supplement the diets.

5.6.2 - Humoral

5.6.2.1 - Lysozyme assay

Fig. 5.8 presents the serum lysozyme activity of turbot as the mean of all individuals tested ± 1 SE. Thirty individuals were tested for the MO-30 treatment, 32 for the MO-300 and CO-30, and 28 for the CO-300 treatment.

No significant differences could be characterised when using the Kruskal-Wallis test ($p < 0.05$) between the dietary treatments.

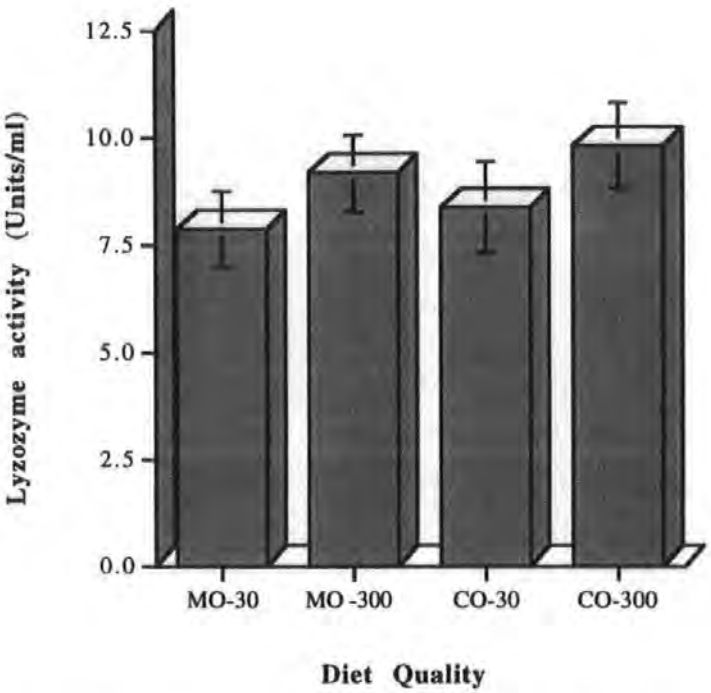


Fig. 5.8: Serum lysozyme activity of turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E and different oil qualities

5.6.2.2 - Protein assay

Fig. 5.9 presents the total serum protein of turbot at the end of the experimental trial. Data are the mean of N individuals ± 1 SE. Twenty nine individuals were tested in fish fed on the MO-30 diet, 33 for the MO-300 diet and CO-30 diet, and 28 for the CO-300 diet.

A significant difference between the various dietary treatments was demonstrated using the Kruskal-Wallis test ($p = 0.0071$). Pairwise comparison tests showed that fish fed with low vitamin E and a high percentage of corn oil had a reduced total serum protein (28.5 mg/ml) compared with fish fed on low levels of vitamin E and a high percentage of marine oil (33.7 mg/ml).

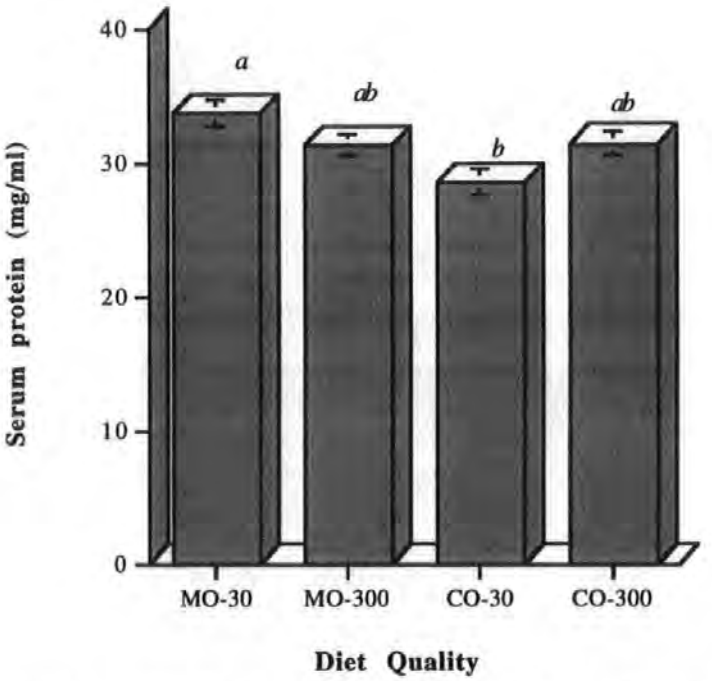


Fig. 5.9: Total serum protein levels of turbot (*S. maximus*) fed for 12 weeks with different levels of vitamin E and different oil qualities. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

5.7 - DISCUSSION

Table 5.9 summarises the parameters showing significant differences between dietary treatments.

Test/Fatty acid	MO-30	MO-300	CO-30	CO-300	STATS
Liver vitamin E (mg/kg)	69 ± 12.8^{a}	270 ± 38^{b}	21.4 ± 5.9^{c}	117 ± 27.8^{a}	$p = 0.0001$
Final length (cm)	12 ± 0.3^{ab}	12.4 ± 0.3^{a}	11.2 ± 0.2^{b}	11.9 ± 0.3^{ab}	$p = 0.022$
Final weight (g)	31.9 ± 2.6^{ab}	35.8 ± 2.8^{a}	24.7 ± 1.8^{b}	31 ± 3^{ab}	$p = 0.0077$
Lymphocyte (%)	37.2 ± 2.7^{a}	48.4 ± 2.4^{b}	42.9 ± 3.5^{ab}	37.4 ± 3.4^{a}	$p = 0.016$
Thrombocytes (%)	41.8 ± 2.2^{a}	39.9 ± 2.2^{a}	42.1 ± 2.3^{ab}	51.2 ± 3^{b}	$p = 0.016$
Proliferation LPS (SI)	0.6 ± 0.1^{a}	1.7 ± 0.4^{b}	0.8 ± 0.09^{a}	2.2 ± 0.8^{b}	$p = 0.0001$
Protein assay (mg/kg)	33.7 ± 1^{a}	31.3 ± 0.9^{ab}	28.5 ± 1^{b}	31.4 ± 0.9^{ab}	$p = 0.0071$

Table 5.9: Summary of significant results found in the experimental trial. Superscript letters denote statistical differences. Values sharing the same superscript are not significantly different

Vitamin E levels measured in the livers of turbot after 12 weeks of feeding showed a good correlation ($R = 0.791$, $p < 0.01$) with dietary vitamin E levels indicating a good uptake. Marine oil fed fish had increased liver vitamin E levels compared with fish fed on corn oil, although the levels added to the diet were identical. This was probably due to the increased levels of vitamin E detected in the diets prepared with marine oil after manufacture compared with levels measured in diets prepared with corn oil (although the differences in the original diets were not significant). This discrepancy between α-tocopherol levels in diets differing in their lipid content was observed previously by Bell *et al.* (1995b), with diets prepared with borage oil and marine oil. Both diets were supplemented with 40 mg of α-tocopherol, but differences were detected in the vitamin E levels after manufacture. The borage oil diet was shown to contain 200 mg/kg α-tocopherol, whereas the marine oil diet contained 350 mg/kg of diet. This could suggest that vitamin E levels in the oil preparation influenced the vitamin E levels in the diet after manufacture or that a component contained in the corn oil somehow induced a degradation of vitamin E included in the ingredients.

At the end of the trial a significant difference ($p < 0.01$) was observed between mean weights of turbot (*Scophthalmus maximus*) fed on different diets. Fish fed with high levels of vitamin E and high percentages of marine oil showed higher mean weights than fish fed on high corn oil and low vitamin E. This suggests that the combination of marine oil and high vitamin E levels was important to promote growth in turbot.

Data on dietary supplementation with vitamin E varies according to the species and the study considered. Watanabe *et al.*, (1970a,b) showed that carp fed deficient vitamin E diets grew less than carp fed on α-tocopherol enriched diets. Hardie *et al.*, (1990) did not demonstrate any differences in growth of Atlantic salmon fed various levels of vitamin E, whereas Thorarinsson *et al.* (1994) demonstrated an increased average final weight in Chinook salmon fed with an increased level of vitamin E compared with salmon fed on low levels of vitamin E. In rainbow trout, Furones *et al.* (1992) did not detect any differences in the growth rate of fish fed on various vitamin E levels, whereas Frischknecht *et al.* (1994) showed that mean final weights of trout fed with vitamin E supplemented diets were higher than mean final weights of trout fed with unsupplemented vitamin E diets. Finally, Wise *et al.* (1993b) did not detect any differences in growth of

catfish fed various levels of vitamin E. Juvenile Korean Rockfish (*Sebastes schlegeli*) showed lower growth when fed vitamin E deprived diets compared with fish fed 20 or 120 or 500 mg of vitamin E per kg of diet for 16 weeks (Bai and Lee, 1998). It was also shown in chapter 3 that juvenile turbot fed with vitamin E depleted diets had lower final mean weights than turbot fed with diets supplemented with 80 or 200 mg of vitamin E/kg of diet. Numerous studies have focused on lipid nutrition of turbot (Cowey *et al.*, 1976a,b; Gatesoupe *et al.*, 1977a, b; Léger *et al.*, 1979; Bell *et al.*, 1985a,b; Castell *et al.*, 1994; Bell *et al.*, 1995a,b,c). It is generally accepted that (n-3) PUFAs are superior to (n-6) PUFAs, and cod liver oil is superior to corn oil for promoting growth in turbot.

However, Castell *et al.* (1994) showed that the optimum growth and survival rates were obtained when juvenile turbot were fed a diet containing 20:4 (n-6) as the only HUFA compared with other combinations of 20:4 (n-6) and 22:6 (n-3). Bell *et al.* (1995b) did not find any differences in final mean weights of juvenile turbot fed for 12 weeks with diets rich either in eicosapentaenoic acid [EPA, 20:5 (n-3)] or γ-linolenic acid [GLA, 18:3 (n-6)].

Finally, Obach (1993) did not characterise any differences in the weight of turbot fed with various vitamin E levels and different sources of lipids. However, fish used in the current study started the experimental period with lower average weights (10 g) than in the study by Obach (55 g). Therefore, the fish may not have had such large reserves in their tissues at the onset of the feeding regime and might have been more sensitive to any imbalance in the ratio of (n-3)/(n-6) PUFAs, as well as any deficit of PUFAs and vitamin E in their diets.

Furthermore, the development of neural tissue is a major feature in juvenile turbot (Linares and Henderson, 1991). The lipids of neural tissues contain very high levels of (n-3) PUFAs particularly 22:6 (n-3). Therefore the demand in those PUFAs may be higher in juvenile turbot than in more mature fish (Linares and Henderson, 1991). This might account for the greater sensitivity to a decrease of (n-3) PUFAs in the diet of the turbot used in the present study compared with the older turbot used by Obach (1993).

A determination of liver fatty acid composition was carried out at the end of the feeding trial by GC. The statistical differences characterised between livers of fish fed different

diets was correlated with differences observed in the composition of the diets fed to those fish. A few fatty acids detected at different levels in the 4 diets did not induce a statistical difference in the livers after 12 weeks feeding. Although the diet did not influence the composition in the liver above the threshold for statistical characterisation, the trend observed in the levels of these fatty acids in the liver paralelled the trend characterised in the diet.

These results corroborate the findings of previous studies showing an influence of dietary lipids on the composition of fatty acids in different organs of turbot (Cowey *et al.*, 1976a,b; Bell *et al.*, 1985a; Castell *et al.*, 1994; Bell *et al.*, 1995a,b,c).

Blood smears showed significant variations in the number of both lymphocytes and thrombocytes between turbot fed different diets. Fish fed with a high percentage of marine oil showed lower thrombocyte counts compared with fish fed on a high percentage of corn oil and high vitamin E levels. In contrast, the proportion of lymphocytes was significantly increased in fish fed on MO-300 compared with fish fed on CO-300 or MO-30.

Previous studies did not characterise any influence of dietary vitamin E (Hardie *et al.*, 1990) or dietary vitamin C (Roberts, *et al.*, 1995) on white blood cell populations in Atlantic salmon or turbot respectively. A difference was seen in the relative proportions of white blood cells in the previous experiments (chapter 3 and 4) but these could be correlated with infection outbreaks (noted at the time, chapter 3 and 4) rather than with the dietary vitamin E levels.

Salmon fed with various (n-3)/(n-6) ratios did not exhibit any variations in the proportions of white blood cells (Thompson *et al.*, 1996). However Obach (1993) observed a substantial drop in the mean concentration of thrombocytes per mm^3 of blood, in turbot fed with cod liver oil and low vitamin E compared with turbot fed on cod liver oil and high vitamin E, or in turbot fed with groundnut oil and low vitamin E compared with turbot fed on groundnut oil and high vitamin E levels. These results suggest an influence of dietary vitamin E on the white blood cell population. In contrast, the present study demonstrates an effect of dietary lipids on the white blood cell population. Indeed, fish fed on marine oil tend to exhibit lower percentages of thrombocytes than fish fed on corn oil.

Thrombocytes are the mammalian platelet-equivalents in fish. In mammals, platelets are derived from megakaryocytes, themselves derived from the pluripotential Colony-Forming Unit (CFU). Colony stimulating factors (CSFs) and interleukins are responsible for the differentiation of CFU along different pathways which gives rise to the different blood cell types. The balance of different CSFs is partially responsible for the proportions of different cell types. Thrombopoietin is considered to be the primary growth factor for regulating megakaryopoiesis and thrombopoiesis in mammals (Kanshansky *et al.*, 1994). Dietary lipids can modulate cytokine production by lymphocytes and macrophages in mammals (Tappia and Grimble, 1994; Caughey *et al.*, 1996; Tappia and Grimble, 1996; Calder, 1997; Tappia *et al.*, 1997). Therefore dietary lipids, via cytokine production, might have a role to play in the modulation of different blood cell proportions. This could be either by influencing the production of cytokines directly involved in haematopoietic processes or by modulation of CSF factors by cytokines produced by the leucocytes.

However, far less is known on thrombopoiesis in fish. Although numerous studies and reviews have described the morphological and functional properties of these cells in various fish species, the mechanisms controlling their genesis remains to be elucidated. Thrombocytes originate in the spleen from prothrombocytes in elasmobranchs (Prea *et al.*, 1990) and Rombout *et al.* (1996) also described the spleen as the thrombopoietic organ in carp. Earlier observations in plaice indicated that spleen was also the site of production of thrombocytes in plaice (Ellis, 1977). The head-kidney has also been described as a site of thrombopoiesis in sea-bass (Esteban *et al.*, 1989) and in *Sparus auratus* (Zuasti and Ferrer, 1989). However the factors controlling the differentiation from prothrombocytes into thrombocytes have not been identified in fish; thus it is impossible to speculate on which mechanisms might be responsible for the increased thrombocyte numbers measured in the present study.

Amongst the three mitogens used to stimulate the proliferation of lymphocytes, only LPS induced significant differences between the four dietary treatments. Lymphocytes of fish fed with high vitamin E levels showed a greater potential to proliferate compared with lymphocytes of fish fed on low vitamin E levels. This was independent of dietary lipid

quality as proliferation of lymphocytes was not different in fish fed MO-30 and CO-30 or in fish fed MO-300 and CO-300.

Stimulation of lymphocyte proliferation by high dietary vitamin E has been reported in homeothermic animals (Tengerdy, 1989; Meydani, 1995) and in rainbow trout (*Oncorhynchus mykiss*) (Verlhac, 1991; N'Doye, 1993, Verlhac and Gabaudan, 1997).

Several mechanisms could account for the modulation of lymphoproliferation by vitamin E and one of them is the influence of vitamin E on cytokine production. Vitamin E reduced the secretion of macrophage migration inhibitory factor (MIF) but did not affect the production of tumour necrosis factor alpha (TNF-α) or IL-6 in rat macrophages (Sakamoto *et al.*, 1998). Supplementation of healthy humans with α-tocopherol significantly decreased the release of IL-1β and TNF-α from LPS-activated monocytes (Devaraj and Jiadal, 1998). Interleukin 2 (IL-2) is the cytokine responsible for the activation of T-lymphocyte proliferation *in vivo* in mammals. Wang *et al.* (1994) showed that vitamin E could restore the production of IL-2 and IFN-γ in T lymphocytes of mice infected with retroviruses. Furthermore, vitamin E enhanced T-cell and B-cell proliferation suppressed by retrovirus infection at 4, 8, 12 but not 16 weeks post infection (Wang *et al.*, 1994). Recently Pighetti *et al.* (1998) showed that splenic leucocytes of vitamin E and selenium deficient rats proliferated less than leucocytes isolated from control rats. However, IL-2 production was measured and did not vary between rats fed different diets. In contrast, mononuclear cells isolated from rats fed vitamin E and selenium deficient diets did not demonstrate any ability to internalize surface-bound transferrin receptor (TfR). The authors proposed that this could be reponsible for the decreased proliferation observed in vitamin E and selenium deficient rats. Indeed, reduction of internalisation of TfR could cause a depletion of intracellular iron stores thereby compromising the ability of cells to enter the S phase of the cell cycle. Unfortunately an investigation of these processes was outside the scope of this study.

Finally, fish fed with high levels of corn oil and low vitamin E levels had lower total serum protein than fish fed with low vitamin E levels and marine oil diets. However, when feeding fish with high levels of vitamin E, variations in dietary lipids did not affect the

total serum protein content of turbot. This suggests a linked action of lipid and vitamin E in the modulation of total serum protein rather than the influence of one factor.

No correlation between serum protein values and dietary polyunsaturated fatty acids (PUFAs) was demonstrated in Atlantic salmon (Thompson *et al.*, 1996). Similarly, no changes in protein concentration could be detected in salmon fed with different levels of vitamin E and different lipid sources (Waagbø *et al.*, 1993a) or in salmon fed with different oils for 12 weeks (Bell *et al.*, 1996). However, Poston *et al.* (1976) observed that elevated plasma protein was found in both EFA and vitamin E deficient salmon. In the present study, fish fed on the different diets grew well and were in good condition at the end of the trial. The group of fish which demonstrated the lowest mean weight at the end of the trial was the group showing the lowest protein level in the serum, but no signs of disease could be observed. Thus it is difficult to attribute a cause to this variation in total serum protein without further tests to measure the different serum proteins.

CHAPTER 6 - GENERAL DISCUSSION

This study has demonstrated that a minimum dietary dose of vitamin E is necessary to ensure optimum growth and health of juvenile turbot (*Scophthalmus maximus*). Furthermore, it showed that lipids are an important dietary component as both lipid quality and quantity can influence not only the requirements for vitamin E but also growth and some immune parameters in juvenile turbot.

In addition to the conventional methods of growth assessment and nutrient uptake, several assays were used in order to assess some immune parameters of juvenile turbot. As the fish in these studies were small with limited haematopoietic tissue from which to extract cells, the methods used were based on the use of microplates which greatly reduce the number of cells necessary for each test. A range of assays were therefore adapted using juvenile turbot and dab (*Limanda limanda*) caught from Plymouth Sound before the start of the experimental trials.

The first experiment looked at the effect of dietary vitamin E on immune parameters and demonstrated that a minimum dose of vitamin E was necessary to ensure normal growth of turbot. Fish fed on a vitamin E deficient diet showed signs of disease, lethargy and melanism at the end of the feeding trial which indicates that vitamin E is also important to maintain good immune defenses and healthy status in turbot. Surprisingly, the haematocrit was increased in turbot fed depleted diets although most previous studies have shown that it is normally decreased in fish fed on vitamin E depleted diets. However, the decrease in haematocrit probably originated in exudative diathesis and may not be informative regarding fish health.

Further tests could be carried out to try and elucidate the mode of action of vitamin E on cellular processes. Vitamin E could be added to culture of cells extracted from kidney of turbot, and the effect on immune processes measured and compared with the changes obtained by dietary means. Membrane vitamin E levels could also be monitored to establish whether any effects on cellular physiology correlates with the membrane vitamin E levels.

In the second experiment the influence of high and low vitamin E levels together with oxidised or fresh oil on immune parameters of turbot was measured. Oxidation of dietary lipids negatively affected not only growth but several of the immune parameters measured, showing that it is important to prevent oxidation of dietary lipids and supplement diets with antioxidants to prevent deterioration of lipid quality and peroxidative damage *in vivo*. The data obtained for the phagocytosis and pinocytosis assays differed suggesting that oxidation of lipids may affect some aspects of the cellular machinery involved in pinocytosis but not in phagocytosis. Further studies focusing the search on some more fundamental mechanisms involved in those cell processes could bring more information on the mechanisms of action of oxidative stress and immune response.

Although TEM did not show any differences in the ultrastucture of the immune cells, future studies could link biochemical tools with goldlabelling to characterise changes in membrane lipids which are not detectable from the ultrastucture alone. This could be advantageous for the second trial. For instance, parinaric acid, a fluorescent PUFA in its normal state, loses its fluorescent properties when the double bond system is oxidised (Van Den Berg *et al.*, 1989). The loss of fluorescence can be monitored as an indication of the peroxidation of parinaric acid. If such a tool was linked to TEM, it could allow a characterisation of peroxidative damage in the membrane and a localisation of damage to certain types of cells or specific membranes within the cells. If the fixation used for TEM does not affect the fluorescent properties of PUFAs in the membrane, this method could then give a good insight into the localisation of peroxidative damage by dietary lipids or vitamin E depletion in haematopoietic tissues.

The effect of dietary lipids with different doses of vitamin E was addressed in the third experiment. Vitamin E enhanced proliferation of lymphocytes stimulated with LPS and showed that vitamin E could be a positive supplement in the diet of turbot. However, even though dietary lipids proved to be important in the growth of turbot, the two combinations of lipids chosen in this study did not influence the phagocytosis, pinocytosis or proliferation of leucocytes after 12 weeks of feeding. This was a surprise as dietary lipids have been shown to affect a range of immune parameters in mammals, as reviewed by Clader (1997) and the phagocytic ability of rainbow trout macrophages (Bowden *et al.*,

1994). Recent studies showed that fish oil supplementation can influence the expression of major histocompatibility complex class II molecules in human monocytes (Hughes *et al.*, 1995) or the level of expression of different adhesion molecules in rat lymphocytes (Sanderson and Calder, 1998) and therefore could have a role in modulation of specific parameters of the immune response. Dietary lipids may influence other aspects of immune function which were not investigated in the present study so a wider range of immune parameters should be investigated in future studies.

Dietary lipids are believed to influence immune response via effects on membrane fluidity and/or modulation of eicosanoid synthesis. Studies have measured membrane fluidity and eicosanoid production in cells extracted from fish fed different diets. Unfortunately studies have not looked at these effects on the same samples. Dietary lipids affect eicosanoid production in different organs of turbot (Bell *et al.*, 1995) and also affect macrophage membrane fluidity in mammals (Tappia *et al.*, 1997) and in fish (Bowden *et al.*, 1994). Measuring the two parameters in a single study would allow a determination of whether modification of physical properties of the membrane and/or modulation of eicosanoid production are at the origin of the observed immunomodulation. If these two mechanisms influence the immune parameters in opposite ways then it is possible that no overall changes are observed and this may explain the lack of influence of lipids in modulation of the parameters in the present study.

The variation of total serum protein is difficult to explain and indicates that further tests measuring the individual proteins, especially immunoglobulins, could provide more information on the mechanisms of immunomodulation.

In conclusion, this study has shown that dietary vitamin E and lipids are important components of the diet and can be used to enhance the immune response of juvenile turbot. However, the mechanisms of action of these two dietary components have not been completely elucidated in fish. The parameters measured in this study may provide a starting point for looking at the mechanisms of action of lipids and vitamin E. Further research focusing on biochemical and molecular processes in the cell should be carried out to investigate these mechanisms.

CHAPTER 7 - REFERENCES

Alvarez-Pellitero, P. & Pinto, R.M., 1987. Some blood parameters in sea-bass, *Dicentrarchus labrax*, infected by bacteria, virus and parasites. *Journal of Fish Biology*, **31**, supplement A, 259–261.

Anderson, A.A., Fletcher, T.C. & Smith, G.M., 1981. Prostaglandin biosynthesis in the skin of plaice, *Pleuronectes platessa* L. *Comparative Biochemistry and Physiology*, **70C**, 195–199.

Anderson, D.P., 1990. Immunological indicators: effects of environmental stress on immune protection and disease outbreaks. *American Fisheries Society Symposium*, **8**, 38–50.

Anderson, D.P., 1996. Environmental factors in fish health: immunological aspects. *Fish Physiology*, **15**, 289–310.

Ashton, I., Clements, K., Barrow, S.E., Secombes, C.J. & Rowley, A.F., 1994. Effects of dietary fatty acids on eicosanoids-generating capacity, fatty acid composition and chemotactic activity of rainbow trout (*Oncorhynchus mykiss*) leucocytes. *Biochemica et Biophysica Acta*, **1214**, 253–262.

Avtalion, R.R., 1981. Environmental control of the immune response in fish. *Critical Reviews in Environmental Control*, **11**(2), 163–188.

Baehner, R.L., Boxer, L.A., Allen, J.M. & Davis, J., 1977. Autooxidation as basis for altered function by polymorphonuclear leukocytes. *Blood*, **50**, 327–335.

Bai, S.C. & Lee, K.-J., 1998. Different levels of dietary DL-α-tocopheryl acetate affect the vitamin E status of juvenile Korean rockfish, *sebastes schlegeli*. *Aquaculture*, **161**, 405-414.

Baker, R.T.M. & Davies, S.J., 1996. Increased production of docosahexaenoic acid (22:6 (n-3) DHA) in catfish nutritionally stressed by the feeding of oxidised oils and the modulatory effect of dietary α-tocopheryl acetate. *Journal of Fish Biology*, **49**, 748-752.

Baker, R.T.M. & Davies, S.J., 1996. Oxidative nutritional stress associated with feeding rancid oils to African catfish, *Clarias gariepinus* (Burchell) and the protective role of α-tocopherol. *Aquaculture Research*, **27**, 795-803.

Baker, R.T.M. & Davies, S.J., 1997. Muscle and hepatic fatty acid profiles and α-tocopherol status in African catfish (*Clarias gariepinus*) given diets varying in oxidative state and vitamin E inclusion level. *Animal Science*, **64**, 187-195.

Bang, H.O., Dyerberg, J. & Hjorne, N., 1976. The composition of food consumed by Greenland Eskimos. *Acta Medica Scandinavica*, **200**, 69–73.

Bang, H.O. & Dyeberg, J., 1980. Lipid metabolism and ischemic heart disease in Greenland Eskimos. *Advances in Nutritional Research*, **3**, 1–22.

Beck, W.S., Liem, K.F. & Simpson, G.G., 1991. *Life, an introduction to biology*, 3rd ed. New York: Harper Collins Publishers.

Becker, W.M. & Deamer, D.W., 1991. *The world of the cell*, 2nd ed. Redwood city, California: The Benjamin-Cummings Publishing Company.

Beitz, D.C. & Hansen, R.G., 1995. *Animal products in human nutrition*, 1st ed. Glasgow, Blackie Academic and Professional publishers.

Bell, J.G. & Cowey, C.B., 1985. Roles of vitamin E and selenium in the prevention of pathologies related to fatty acid oxidation in salmonids. *Nutrition and feeding in fish*, pp. 333-347, Place: Academic Press, London.

Bell, J.G., Ashton, I., Secombes, C.J., Wetzel, B.R., Dick, J.R. & Sargent, J.R., 1996. Dietary lipid affects phospholipid fatty acid compositions, eicosanoid production and immune function in Atlantic salmon (*Salmo salar*). *Prostaglandins, Leukotrienes and Essential Fatty Acids*, **54**(3), 173–182.

Bell, J.G., Castell, J.D., Tocher, D.R., MacDonald, F.M. & Sargent, J.R., 1995a. Effects of different dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid fatty acid compositions and prostaglandin production in juvenile turbot (*Scophthalmus maximus*). *Fish Physiology and Biochemistry*, **14**(2), 139–151.

Bell, J.G., Tocher, D.R., MacDonald, F.M. & Sargent, J.R., 1995b. Effects of dietary borage oil [enriched in γ-linolenic acid, 18:3(n-6)] or marine fish oil [enriched in eicosapentaenoic acid, 20:5(n-3)] on growth, mortalities, liver histopathology and lipid composition of juvenile turbot (*Scophthalmus maximus*). *Fish Physiology and Biochemistry*, **14**(5), 373–383.

Bell, J.G., Tocher, D.R., MacDonald, F.M. & Sargent, J.R., 1995c. Diets rich in eicosapentaenoic acid and γ-linolenic acid affect phospholipid fatty acid composition and production of prostaglandins E_1, E_2, and E_3 in turbot (*Scophthalmus maximus*), a species deficient in Δ5 fatty acid desaturase. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, **53**, 279–286.

Bell, M.V., Henderson, R.J. & Sargent, J.R., 1985a. Changes in the fatty acid composition of phospholipids from turbot (*Scophthalmus maximus*) in relation to dietary polyunsaturated fatty acid deficiencies. *Comparative Biochemistry and Physiology*, **81B**, 193–198.

Bell, M.V., Henderson, R.J., Pirie, B.J.S. & Sargent, J.R., 1985b. Effects of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gills structure in the turbot, *Scophthalmus maximus*. *Journal of Fish Biology*, **26**, 181–191.

Bell, M.V., Henderson, R.J. & Sargent, J.R., 1986. Mini review: the role of polyunsaturated fatty acids in fish. *Comparative Biochemistry and Physiology*, **83B**, 711–719.

Bender, D.A., 1992. *Nutritional Biochemistry of the vitamins.* Cambridge: Cambridge University Press.

Bendich, A., 1988. Antioxidant vitamins and immune response. In *Nutrition and immunology* (ed. R.K. Chandra), pp. 125–147. New York: Alan R. Liss Inc.

Bendich, A., 1989. The effect of vitamin E on the immunosuppressive effects of (n-6) polyunsaturated fatty acids. *Annals of the New York Academy of Sciences*, **570**, 412–414.

Bendich,A., Gabriel, E. & Machlin, L.J., 1986. Dietary vitamin E requirement for optimum immune responses in the rat. *Journal of Nutrition*, **116**(4), 675–681.

Bensoussan, K., Morales, C.R. & Hermo, L., 1998. Vitamin E deficiency causes incomplete spermatogenesis and affects differentiation of epithelial cells of the epididymis in the rat. *Journal of Andology*, **19**(3), 266-288.

Berg, J.J.M. van den, Roefolsen, B., Op Den Kamp, J.A.F. & Deenen, L.L.M. van, 1989. Cooperative protection by vitamins E and C of human erythrocyte membranes against peroxidation. *Annals of the New York Academy of Sciences*, **570**, 527–529.

Blazer, V.S., 1991. Piscine macrophages function and nutritional influences: a review. *Journal of Aquatic Animal Health*, **3**, 77–86.

Blazer, V.S. & Wolke, R.E. 1984. The effects of α-tocopherol on the immune response and non specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). *Aquaculture*, **37**, 1–9.

Bly, J.E., Buttke, T.M. & Clem, L.W., 1990. Differential effects of temperature and exogenous fatty acids on mitogen-induced proliferation in channel catfish T and B lymphocytes. *Comparative Biochemistry and Physiology*, **95A**(3), 417-424.

Borenfreund, E. & Puerner, J., 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters*, **24**, 119–124.

Bowden, L.A., Weitzel, B., Ashton, I.P., Secombes, C.J., Restall, C.J., Walton, T.J.& Rowley, A.F., 1994. Effect of dietary cholesterol on membrane properties and immune functions in rainbow trout. *Biochemical Society Transactions*, **22**, 339S.

Bruno, D.W., 1986. Changes in serum parameters of rainbow trout, *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L., infected with *Renibacterium-salmoninarum*. *Journal of Fish Diseases*, **9**(3), 205–211.

Bruno, D.W. & Munro, A.L.S., 1986. Hematological assessment of rainbow trout, *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L., infected with *Renibacterium-salmoninarum*. *Journal of Fish Diseases*, **9**(3), 195–204.

Burton, G.W., 1989. Antioxidant actions of carotenoids. *Journal of Nutrition*, **119**, 109–111.

Burton, G.W. & Ingold, K.U., 1981. Autoxidation of biological molecules: (i) the antioxidant activity of vitamin E and related chain breaking phenolic antioxidants *in vitro*. *Journal of the American Chemical Society*, **103**, 6472–6477.

Burton, G.W. & Ingold, K.U., 1984. β-carotene, an unusual type of lipid antioxidant. *Science, New York*, **224**, 569–573.

Burton, G.W., Joyce, A. & Ingold, K.U., 1982. First proof that vitamin E is a major lipid soluble, chain breaking antioxidant in human blood plasma. *The Lancet*, **II**, 327.

Burton, G.W., Joyce, A. & Ingold, K.U., 1983. Is vitamin E the only lipid-soluble, chain breaking antioxidant in human blood plasma and erythrocyte membranes? *Archives of Biochemistry and Biophysics*, **221**(1), 281–290.

Calder, P.C., 1993. The effects of fatty acids on lymphocyte functions. *Brazillian Journal of Medical and Biological Research*, **26**, 901–917.

Calder, P.C., 1997. N-3 polyunsaturated fatty acids and immune function. *Advances in Enzyme Regulation*, **37**, 197-237.

Calder, P.C., Bevan, S.J. & Newsholme, E.A., 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. *Immunology*, **75**, 108–115.

Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S. & Newsholme, E.A., 1990a. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochemical Journal*, **269**, 807–814.

Calder, P.C., Bond, J.A. & Newsholme, E.A., 1990b. Fatty acids inhibition of lipopolysaccharide-stimulated B lymphocyte proliferation. *Biochemical Society Transactions*, **18**, 904–905.

Calder, P.C. & Newsholme, E.A., 1993b. Effects of omega-3 fatty acids on lymphocyte functions. In *Omega-3 fatty acids: metabolism and biological effects* (ed. C.A. Drevon, C.A. Basksass, I. Krokan), pp. 293-303.

Castell, J.D., Bell, J.G., Tocher, D.R. & Sargent, J.R., 1994. Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). *Aquaculture*, **128**, 315–333.

Castell, J.D., Sinnhuber, R.O., Wales, J.H. & Lee, D.J., 1972. Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): growth, feed conversion and some gross deficiency symptoms. *Journal of Nutrition*, **102**(1), 77–85.

Caughey, G.E., Mautzioris, E., Gibson, R.A., Cleland, L.G. & James, M, 1996. The effect on human tumor necrosis factor α and interleukin 1β production of diets enriched in (n-3) fatty acids from vegetable oil or fish oil. *American Journal of Clinical Nutrition*, **63**, 116-122.

Cheeseman, K.H., Burton, G.W., Ingold, K.U. & Slater, T.F., 1984. Lipid peroxidation and lipid antioxidant in normal and tumour cells. *Toxicologic Pathology*, **12**, 235–239.

Cheeseman, K.H., Emery, S., Maddix, P., Slater, T.F., Burton, G.W. & Ingold, K.U., 1988. Studies on lipid peroxidation in normal and tumour tissues – the Yoshida rat-liver tumor. *Biochemical Journal*, **250**(1), 247–252.

Christiansen, R., Glette, J., Lie, O., Torrissen, O.J. & Waagbo, R., 1995. Antioxidant status and immunity in Atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. *Journal of Fish Diseases*, **1995**(18), 317–328.

Claesson, H.-E., Odlander, B. & Jakobsson, P.-J., 1992. Leukotriene B_4 in the immune system. *International Journal of Immunopharmacology*, **14**, 441–449.

Cohn, W. & Kuhn, H., 1989. The role of low-density-lipoprotein receptor for α-tocopherol delivery in tissues. *Annals of the New York Academy of Sciences*, **570**, 61–71.

Cossins, A.R., Friedlander, M.J. & Prosser, C.D., 1977. Correlations between behavioral temperature adaptations of goldfish and the viscosity and fatty acid composition of their synaptic membranes. *Journal of Comparative Physiology* A, **120**, 109–121.

Cowey, C.B., Adron, J.W., Owen, J.M. & Roberts, R.J., 1976a. The effects of different dietary oils on tissue fatty acids and tissue pathology in turbot (*Scophthalmus maximus*). *Comparative Biochemistry and Physiology*, **53B**, 399–403.

Cowey, C.B., Owen, J.M., Adron, J.W. & Middleton, C., 1976b. Studies on the nutrition of marine flatfish. The effect of different dietary fatty acids on the growth and fatty acid composition of turbot (*Scophthalmus maximus*). *British Journal of Nutrition*, **36**, 479–486.

Deniel, C., 1974. Régime alimentaire des jeunes turbots (*Scophthalmus maximus*) de la classe O dans leur milieu naturel. *Cahiers de Biologie Marine*, **15**, 551–588.

Devaraj, S. & Jialal, I., 1998. The effects of α-tocopherol on critical cells in artherogenesis. *Current Opinion in Lipidology*, **9**, 11-15.

Diplock, A.T. & Lucy, J.A., 1973. The biochemical modes of action of vitamin E and selenium, a hypothesis. *FEBS Letters*, **29**, 205–210.

Dushkin, M., Schwartz, Y., Volsky, N., Musatov, M., Vereschagin, E., Ragino, J., Perminova, O. & Kozlov, V., 1998. Effects of oxysterol upon macrophages and lymphocytes functions in vitro. *Prostaglandins and Other Lipid Mediators*, **55**, 219-236.

Duswald, K.H., 1985. *Zur pathobiochemie der leukozyten-elastase und ausgewahlter plasmaproteine bei sepsis nach abdominalchriturgische eingriffen*. Darmstadt, Germany: Ernst Giebeler G-I-T Verlag.

Ellis, A.E., 1977. The leukocytes of fish: A review. *Journal of Fish Biology*, **11**, 453-491.

Ellis, A.E., 1981. Stress and the modulation of defence mechanisms in fish. In *Stress and fish* (ed. A.D. Pickering), pp. 147–169. London: Academic Press.

Ellis, A.E. & Munroe, A.L.S., 1976. Defence mechanisms in fish. 1. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice (*Pleuronectes platessa* L.). *Journal of Fish Biology*, **8**, 67–78.

Erdal, J.I., Evensen, O., Kaurstad, O.K., Lillehaug, A., Solbaken, R. & Thorud, K., 1991. Relationship between diet and immune response in Atlantic salmon (*Salmo salar L.*) after feeding various levels of ascorbic acid and omega-3 fatty acids. *Aquaculture*, **98**, 363–379.

Eskew, M.L., Scholz, R.W., Reddy, C.C., Todhunter, D.A. & Zarkower, A., 1985. Effect of vitamin E and selenium deficiencies on rat immune functions. *Immunology*, **54**, 173–180.

Esteban, M.A., Meseguer, J., Garcia Ayala & A., Agulleiro, B., 1989. Erythropoiesis and thrombopoiesis in the head-kidney of the sea-bass (*Dicentrarchus labrax* L.): an ultrastructural study. *Archives of Histology and Cytology*, **52**(4), 407-419.

Evans, H.M. & Bishop, K.S., 1922. On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science, New York*, **56** (1458), 650–651.

Evenberg, D., De Graaff, P., Fleuren, W. & Van Muiswinkel, W., 1986. Blood changes in carp (*Cyprinus carpio*) induced by ulcerative *Aeromonas salmonicida* infections. *Veterinary Immunology and Immunopathology*, **12**, 321–330.

Fänge, R., 1992. Fish blood cells. In *Fish physiology, the cardiovascular system* (ed. W.S. Hoar, D.J. Randall & A.P. Farrell), pp. 1–54. Academic Press Inc., vol. 12, Part B.

Fänge, R., Lundbald, G. & Lind, J., 1976. Lysozyme and chitinase in blood and lymphomyeloid tissues of marine fish. *Marine Biology*, **36**, 277-282.

Fletcher, T.C. & White, A., 1973. Lysozyme activity in the plaice (*Pleuronectes platessa* L.) *Experientia*, **29**, 1283-1285.

Forster, I., Higgs, D.A., Bell, G.R., Dosanjh, B.S. & March, B.E., 1988. Effect of diets containing herring oil oxidized to different degrees on growth and immunocompetence of juvenile coho salmon (*Oncorhynchus kisutch*). *Canadian Journal of Fisheries and Aquatic Sciences*, **45**, 2187–2194.

Fracalossi, D.M. & Lovell, R.T., 1994. Dietary lipid sources influence responses of channel catfish (*Ictalurus punctatus*) to challenge with the pathogen *Edwarsellia ictaluri*. *Aquaculture*, **119**, 287–298.

Frischknecht, R., Wahli, T. & Meier, W., 1994. Comparison of pathological changes due to deficiency of vitamin C, vitamin E and combinations of vitamins C and E in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, **17**, 31–45.

Furones, M.D., Alderman, D.J., Bucke, D., Fletcher, T.C., Knox, D. & White, A., 1992. Dietary vitamin E and the response of rainbow trout, *Oncorhynchus mykiss* (Walbaum), to infection with *Yersinia ruckeri*. *Journal of Fish Biology*, **41**, 1037–1041.

Gallet de Saint Aurin, D., 1987. Pathologie du loup (*Dicentrarchus labrax*) en élevage intensif en Martinique. In *Proceedings of the 38th Annual Gulf and Carribbean Fisheries Institute, Miami, Florida, May* (ed. F. Williams), pp. 144–163. Miami, Florida.

Gatesoupe, F.J., Léger, C., Métailler, R. & Luquet, P., 1977a. Alimentation lipidique du turbot (*Scophthalmus maximus* L.). I. Influence de la longueur de chaîne des acides gras de la série ω3. *Annales Hydrobiologiques*, **8** (1), 89–97.

Gatesoupe, F.J., Léger, C., Boudon, M., Métailler, R. & Luquet, P., 1977b. Alimentation lipidique du turbot (*Scophthalmus maximus* L.). II. Influence de la supplémentation en esters méthyliques de l'acide linolénique et de la complémentation en acides gras de la série ω 9 sur la croissance. *Annales Hydrobiologiques*, **8** (2), 247–254.

Gatlin, D.M., Bai, S.C. & Erickson, M.C., 1992. Effects of dietary vitamin E and synthetic antioxidants on composition and storage quality of channel catfish, *Ictalurus punctatus*. *Aquaculture*, **106** (3/4), 323–332.

Gershwin, M.E., Beach, S. & Hurley, L.S., 1985. *Lipids, nutrition and immunity* pp. 259–284. New York: Academic Press.

Goetzl, E.J., 1980. Vitamin E modulates the lipoxygenation of arachidonic acid in leukocytes. *Nature, London*, **280**, 183–185.

Gomez-Fernandez, J.C., Villalain, J., Aranda, F.J., Ortiz, A., Micol, V., Couthino, A., Berberan-Santos, M.N. & Prieto, M.J.E., 1989. Localization of alpha-tocopherol in membranes. *Annals of the New York Academy of Sciences*, **570**, 109–120.

Greene, D.H.S. & Selivonchick, D.P., 1987. Lipid metabolism in fish. *Progress Lipid Research*, **26**, 53-85.

Grinde, B., 1989. Lysozyme from rainbow trout *Salmo gairdneri* Richardson as an antibacterial agent against fish pathogens. *Journal of Fish Diseases*, **12**, 95–104.

Halliwell, B. & Gutteridge, J.M.C., 1985. *Free radicals in biology and medicine*. Place: Oxford Science Publications.

Halver, J.E., 1972. *Fish nutrition*. London: Academic Press.

Hardie, L.J., 1991. *The effect of dietary vitamins, lipids and temperature on teleost immunity*. PhD thesis, University of Aberdeen, Scotland.

Hardie, L.J., Fletcher, T.C. & Secombes, C.J., 1990. The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.). *Aquaculture*, **87**, 1–13.

Harman, D., 1982. The free-radical theory of ageing. In *Free radicals in biology* (ed. W.A. Pryor), p. 255. London: Academic Press.

Hashimoto, Y., Okaichi, T., Watanabe, T., Furukawa, A. & Umezu, I., 1966. Muscle distrophy of carp due to oxidised oil and the preventive effect of vitamin E. *Bulletin of the Japanese Society of Scientific Fisheries*, **32**(1), 64-69.

Henderson, R.J., Bell, M.V. & Sargent, J.R., 1985. The conversion of polyunsaturated fatty acids to prostaglandins by tissue homogenates of the turbot, *Scophthalmus maximus* (L.). *Journal of Experimental Marine Biology and Ecology*, **85**, 93–99.

Hesse, D., Keller, H.E., Oberlin, B., Bonfanti, R. & Schep, W., 1991. Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase. *International Journal of Vitamins and Nutrition Research*, **61** (3), 232–238.

Hines, R. & Spira, D.T., 1973. Ichthyophthyriasis in the mirror carp. III. Leukocytes response. *Journal of Fish Biology*, **5**, 527–534.

Hughes, D.A., Pinder, A.C., Piper, Z., Johnson, I.T. & Lund, E.K., 1996. Fish oil supplementation inhibits the expression of major histocompatibility complex class II molecules and adhesion molecules on human monocytes. *American Journal of Clinical Nutrition*, **63**, 267–272.

Hung, S.S.O., Walker, B.L. & Slinger, S.L., 1983. Effect of oxidised oil on the liver fatty acids of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology*, **76B**(2), 349-353.

Iwama, G. & Nakanishi, T., 1996. *The fish immune system. Organism, pathogen, and environment.* London: Academic Press.

Iwama, G.K., Greer, G.L. & Randall, D.J., 1986. Changes in selected haematological parameters in juvenile Chinook salmon subjected to a bacterial challenge and a toxicant. *Journal of Fish Biology*, **28**, 563–572.

Jakobsson, P.-J., Steinhilber, D., Odlander, B., Radmark, O., Claesson, H.-E. & Samuelsson, B., 1992. On the expression and regulation of 5-lipoxygenase in human lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 3521–3525.

Johnston, P.V., 1988. Lipid modulation of immune responses. In *Nutrition and Immunology* (ed. R.K. Chandra), pp. 37–86. New York: Alan R. Liss, Inc.

Jollivet, D., Gabaudan, J. & Métailler, R., 1988. Some effects of physical state and dietary level of starch, temperature and meal size on turbot (*Scophthalmus maximus* L.) digestive processes. *International Council for the Exploitation of the Sea (CM Papers and Reports) Mariculture Commitee*, CM 1988/F:25.

Jones, A., 1970. *Some aspects of the biology of the turbot* Scophthalmus maximus *L. with special reference to feeding and growth in the juvenile stage*. PhD thesis, University of East Anglia.

Kagan, V.E., 1989. Tocopherol stabilizes membrane against phospholipase A, free fatty acids and lysophospholipids. *Annals of the New York Academy of Sciences*, **570**(Dec.), 121–135.

Kanazawa, K., Kanazawa, E. & Natake, M., 1985. Uptake and secondary autooxidation products of linoleic acid by the rat. *Lipids*, **20** (7), 412–419.

Kanazawa, K., Danno, G.-I. & Natake, M., 1975. Lysozyme damage caused by secondary degradation products during the autoxidation process of linoleic acid. *Journal of Nutritional Science and Vitaminology*, **21**, 373-382.

Kaneda, T. & Miyazawa, T., 1987. Lipid peroxides and nutrition. *World Review on Nutrition and Diet*, **50**, 186-214.

Kanshanski, K., Lok, S., Holly, R.D., Broudy, V.C., Lin, N., Bailey, M.C., Forstrom, J.W., Buddle, M.M., Oort, P.J., Hagen, F.S., Roth, G.J., Papayannopoulou, T. & Foster, D.C., 1994. Promotion of megakariocyte progenitor expansion and differientiation by the c-Mpl ligand thrombopoietin. *Nature*, **369**, 568-571.

Kiron, V., Fukuda, H., Takeuchi, T. & Watanabe, T., 1995. Essential fatty acid nutrition and defence mechanisms in rainbow trout *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology*, **111A** (3), 361–367.

Kline, K., Cochran, G.S., Huggins, E.R., Morgan, T.J., Rao, A. & Sanders, B.G., 1989. Vitamin E modulation of retrovirus-induced immune dysfunctions and tumor cell proliferation. *Annals of the New York Academy of Sciences*, **570**, 470–473.

Knight, J., Lloyd-Evans, P., Rowley, A.F. & Barrow, S.E., 1995. Effect of lipoxins and other eicosanoids on phagocytosis and intracellular calcium mobilisation in rainbow trout (*Oncorhynchus mykiss*) leukocytes. *Journal of Leukocyte Biology*, **54**, 518-522.

Knight, J. & Rowley, A.F., 1995. Immunoregulatory activities of eicosanoids in the rainbow trout, *Oncorhynchus mykiss*. *Immunology*, **85**, 389-393.

Koven, W.M., Henderson, R.J. & Sargent, J.R., 1994. Lipid digestion in turbot (*Scophthalmus maximus*). I. Lipid class and fatty acid composition of digesta from different segments of the digestive tract. *Fish Physiology and Biochemistry*, **13** (1), 69–79.

Kromann, N. & Green, A., 1980. Epidemiological studies in the Upernavik District, Greenland. *Acta Medica Scandinavica*, **208**, 401–406.

Kruskal, W.H. & Wallis, W.A., 1952. Use of ranks in one-criterion analysis of variance. *Journal of American Statistics Association*, **47**, 583–621.

Lall, S.P., Olivier, G., Hines, J.A. & Ferguson, H.W., 1988. The role of vitamin E in nutrition and immune response of Atlantic salmon (*Salmo salar*). *Bulletin of Aquaculture Association of Canada*, **88**(2), 76–78.

Laudan, R., Stolen, J. & Cali, A., 1986. Immunoglobulin levels of the winter flounder (*Pseudopleuronectes americanus*) and the summer flounder (*Paralichthys dendatus*) injected with the microsporidian parasite *Glugea stepheni*. *Developmental & Comparative immunology*, **10**, 331-340.

Lee, T.H., Hoover, R.L., Williams, J.D., Sperling, R.I., Ravalese III, J., Spur, B.W., Robinson, D.R., Corey, E.J., Lewis, R.A. & Austen, F.K., 1985. Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on *in vitro* neutrophil and monocyte leukotriene generation and neutrophil function. *The New England Journal of Medicine*, **312** (19), 1217–1224.

Léger, C., Gatesoupe, F.-J., Métailler, R., Luquet, P. & Fremont, L., 1979. Effect of dietary fatty acids differing by chain lengths and ω series on the growth and lipid composition of turbot *Scophthalmus maximus* L. *Comparative Biochemistry and Physiology*, **64B**, 345–350.

Linares, F. & Henderson, R.J., 1991. Incorporation of ^{14}C-labelled polyunsaturated fatty acids by juvenile turbot, *Scophthalmus maximus* (L.) *in vivo*. *Journal of Fish Biology*, **38**, 335–347.

Lindsay, G.J.H., 1986. The significance of chitinolytic enzymes and lysozyme in rainbow trout (*Salmo gairdneri*) defence. *Aquaculture*, **51**, 169-173

Liu, J-F. & Lee, Y-W., 1998. Vitamin C supplementation restores the impaired vitamin E status of guinea pigs fed oxidised frying oil. *The Journal of Nutrition*, **128**, 116-122.

Lloyd-Evans, P., Barrow, S.E., Hill, D.J., Bowden, L.A., Rainger, G.E., Knight, J. & Rowley, A.F., 1994. Eicosanoid generation and effects on the aggregation of thrombocytes from the rainbow trout, *Oncorhynchus mykiss. Biochemica et Biophysica Acta*, **1215**, 291-299.

Maguire, J.J., Wilson, D.S. & Packer, L., 1989. Mitochondrial electron transport-linked tocopheroxyl radical reduction. *Journal of Biological Chemistry*, **264** (21), 462–465.

Manz, U. & Philipp, K., 1988. Determination of α-tocopherol in complete feeds, premixes and vitamin concentrates with the aid of HPLC. In *Analytical methods for vitamins and carotenoid in feed* (ed. H.E. Keller), no. 2101, pp. 12–13. Basle, Switzerland: Hoffmann-LaRoche Ltd.

May, J.M., Qu, Z-C. & Mendiratta, S., 1998. Protection and recycling of α-tocopherol in human erythrocytes by intracellular ascorbic acid. *Archives of Biochemistry and Biophysics*, **349**(2), 281-289.

Messager, J.-L., Stephan, G., Quentel, C. & Baudin Laurencin, F., 1992. Effects of dietary oxidized fish oil and antioxidant deficiency on histopathology, haematology, tissue and plasma biochemistry of sea-bass *Dicentrarchus labrax. Aquatic Living Resources*, **5**, 205–214.

Meydani, M., 1995. Vitamin E. *The Lancet*, **345**, 170-175.

Meydani, S.N., Meydani, M., Barklund, P.M., Liu, S., Miller, R.A., Cannon, J.C., Rocklin, R. & Blumberg, J.B., 1989. Effect of vitamin E supplementation on immune responsiveness of the aged. *Annals of the New York Academy of Sciences*, **570**, 283–289.

Meydani, S.N., Meydani, M., Blumberg, J.B., Leka, L.S., Siber, G., Loszewski, R., Thompson, C., Pedrosa, M.C., Diamond, R.D. Stollar, D., 1997. Vitamin E supplementation and *in vivo* immune response in healthy elderly subjects. A randomised control trial. *JAMA*, **277**, 1380-1386.

Miki, M., Motoyama, T. & Mino, M., 1989. Synergistic inhibition of oxidation in RBC ghosts by vitamins C and E. *Annals of the New York Academy of Sciences*, **570**, 474–477.

Moccia, R.D., Hung, S.S.O., Slinger, S.J. & Ferguson, H.W., 1984. Effect of oxidised fish oil, vitamin E and ethoxyquin on the histopathology of rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases*, **7**, 269–282.

Murray, C.K. & Fletcher, T.C., 1976. The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. *Journal of Fish Biology*, **9**, 329-334.

N'Doye, A.T., 1993. *Etude des effets* in vivo *et* in vitro *de la vitamine E sur la fonction immune de la truite arc-en-ciel* (Oncorhynchus mykiss). Thèse pour le diplôme de docteur de l'université de Limoges, France.

Nockels, C.F., 1979. Protective effects of supplemental vitamin E against infection. Roche Information Service, Animal Nutrition Department. *Reprint. Federation Proceedings*, **38**, 2134–2138.

Norman, J.R., 1934. *A systematic monograph of the flatfishes (heterosomata).* Vol. 1. *Psettodidae, bothidae, pleuronectidae*. Place: British National Museum Publications.

Oarada, M., Miyazawa, T. & Kaneda, T., 1986. Distribution of ^{14}C after oral administration of (U-^{14}C) labelled methyl linoleate hydroperoxides and their secondary oxidation products in rats. *Lipids*, **21** (2), 150–154.

Obach, A., 1993. *Influence de différents facteurs de l'environnement sur certains aspects de l'immunité chez le turbot* Scophthalmus maximus *L., le bar* Dicentrarchus labrax *L., et la daurade* Sparus aurata *L.* Thèse de Doctorat de l'Université de Bretagne occidentale (France), spécialité océanologie biologique, option aquaculture et pêche.

Obach, A. & Baudin Laurencin, F., 1992. Effects of dietary oxidised fish oil and deficiency of anti-oxidants on the immune response of turbot, *Scophthalmus maximus*. *Aquaculture*, **107**, 221–227.

Obach, A., Quentel, C. & Baudin Laurencin, F., 1993. Effects of alpha-tocopherol and dietary oxidised fish oil on the immune response of sea bass *Dicentrarchus labrax*. *Diseases of Aquatic Organisms*, **15**, 175–185.

Ogata, H., Nomura, T. & Hata, M., 1978. Prostaglandin biosynthesis in the tissue homogenates of marine animals. *Bulletin of the Japanese Society of Scientific Fisheries*, **44** (12), 1367–1370.

Owen, J.M., Adron, J.W., Sargent, J.R. & Cowey, C.B., 1972. Studies on the nutrition of marine flatfish. The effect of dietary fatty acids on the tissue fatty-acids of the plaice *Pleuronectes platessa*. *Marine Biology*, **13**, 160–166.

Owen, J.M., Adron, J.W., Middleton, C. & Cowey, C.B., 1975. Elongation and desaturation of dietary fatty acids in turbot *Scophthalmus maximus* L., and rainbow trout, *Salmo gairdneri* Richardson. *Lipids*, **10** (9), 528–531.

Packer, L. & Landvick, S., 1989. Vitamin E - introduction to biochemistry and health benefits. *Annals of the New York Academy of Sciences*, **570**, 1–6.

Panush, R.S. & Delafuente, J.C., 1985. Vitamins and immunocompetence. *World Review of Nutrition and Dietetics*, **45**, 97–132.

Peck, M.D., 1994. Interaction of lipids with immune function. I. Biochemical effects of dietary lipids on plasma membranes. *Journal of Nutritional Biochemistry*, **5**, 466–478.

Person-Le Ruyet, J., 1990. Sole and turbot culture. In *Aquaculture*, vol. 2, (ed G. Barnabe), pp. 687–734. Chichester, West Sussex: Ellis Horwood Ltd.

Peters, G. & Schwarzer, R., 1985. Changes in haemopoietic tissue of rainbow trout under influence of stress. *Diseases of Aquatic Organisms*, **1**, 1–10.

Pettitt, T.R., Rowley, A.F., Barrow, S.E., Mallet, A.I. & Secombes, C.J., 1991. Synthesis of lipoxins and other lipoxygenase products by macrophages from the rainbow trout *Oncorhynchus mykiss*. *Journal of Biological Chemistry*, **266** (14), 8720–8726.

Pica, A., Lodato, A., Grimaldi, M.C. & Della Corte, F., 1990. Morphology, origin, and functions of the thrombocytes of elasmobranchs. *Archivio Italiano di Anatomia e di Embriologia*, **95**(3/4), 187-207.

Pickford, G.E., Srivastava, A.K., Slicher, A.M. & Pang, P.K.T., 1971. The stress response in the abundance of circulating leucocytes in the killifish, *Fundulus heteroclitus*. III. The role of the adrenal cortex and a concluding discussion of the leucocyte-stress syndrome. *Journal of Experimental Zoology*, **177**, 109–118.

Pighetti, G.M., Eskew, M.L., Reddy, C.C. & Sordillo, L.M., 1998. Selenium and vitamin E deficiency impair transferrin receptor internalisation but not IL-2, IL-2 receptor, or transferrin receptor expression. *Journal of Leukocyte Biology*, **63**, 131-137.

Pipe, R.K., Coles, J.A. & Farley, S.R., 1995. Assays for measuring immune response in the mussel *Mytilus edulis*. *Techniques in Fish Immunology*, **4**, 93–100.

Poston, H.A., Combs, G.F. & Leibovitz, L., 1976. Vitamin E and selenium interrelations in the diet of Atlantic salmon (*Salmo salar*): gross, histological and biochemical deficiency. *Journal of Nutrition*, **106**, 892–904.

Prasad, J.S., 1980. Effect of vitamin E supplementation on leukocyte function. *American Journal of Clinical Nutrition*, **33**, 606–608.

Pulsford, A., Crampe, M., Langston, A. & Glynn, P.J., 1995. Modulatory effects of disease, stress, copper, TBT, and vitamin E on the immune system of flatfish. *Fish and Shellfish Immunology*, **5**, 631–643.

Pulsford, A., Lemaire-Gony, S. & Farley, S., 1992. Effects of environmental stress on dab *Limanda limanda* immune system. *International Council for the Exploration of the Sea (CM Papers and Reports) Marine Environmental Quality Commitee*, CM 1992/E:14.

Rabinovich, M., 1995. Professional and non-professional phagocytes: an introduction. *Trends in Cell Biology*, **5**, 85-87.

Rainger, G.E., Rowley, A.F. & Pettitt, T.R., 1992. Effect of inhibitors of eicosanoid biosynthesis on the immune reactivity of the rainbow trout, *Oncorhynchus mykiss*. *Fish & Shellfish Immunology*, **2**, 143-154.

Raymond, J.C., 1988. *Syndrome de cécité-mélanisme chez trois espèces de poissons marins élevés en cages flottantes à la Martinique,* Ocyurus chrysurus, Lutjanus analis *et* Dicentrarchus labrax. Thèse pour le diplôme d' Etat de Docteur Vétérinaire, France.

Repine, J.E., Rao, G., Beall, G.D. & White, J.G., 1978. Inhibition of human neutrophil oxidative metabolism and degranulation *in vitro* by nitroblue tetrazolium and vitamin E. *American Journal of Pathology*, **90**, 659–674.

Ricker, W.E., 1979. Growth rates and models. *Fish Physiology*, **8**, 677–743.

Roberts, M.L., Davies, S.J. & Pulsford, A.L., 1995. The influence of ascorbic acid (vitamin C) on non-specific immunity in the turbot. *Fish & Shellfish Immunology*, **5**, 27–38.

Roem, A.J., Kohler, C.C. & Stickney, R.R., 1990. Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. *Aquaculture*, **87**, 155–164.

Rombout, J.H.W.M., Koumans-Van Diepen, J.C.E., Emmer, P.M., Taverne-Thiele, J.J. & Taverne, N., 1996. Characterisation of carp thrombocytes with specific monoclonal antibodies. *Journal of Fish Biology*, **49**, 521-531.

Rowley, A.F., Lloyd-Evans, P., Barrow, S.E. & Serhan, C.N., 1994. Lipoxin biosynthesis by trout macrophages involves the formation of epoxide intermediates. *Biochemistry*, **33** (4), 856–863.

Rowley, A.F., Knight, J., Lloyd-Evans, P., Holland, J.W. & Vickers, P.J., 1995. Eicosanoids and their role in immune modulation in fish – a brief overview. *Fish and Shellfish Immunology*, **5**, 549–567.

Sakamoto, W., Fujie, K., Nishihira, J., Handa, H., Ueda, N. & Yamamoto, S., 1996. Effect of vitamin E on expression of cyclooxygenase-2 in lipopolysaccharide-stimulated rat macrophages. *Biochemica et Biophysica Acta*, **1304**, 139-144.

Sakamoto, W., Nishihira, J., Fujie, K., Handa, H., Ozaki, M. & Yukawa, S., 1998. Inhibition of macrophage migration inhibitory factor secretion from macrophages by vitamin E. *Biochemica et Biophysica Acta*, **1404**, 427-434.

Salhi, M., Izquierdo, M.S., Hernandez-Cruz, C.M., Gonzalez, M. & Fernandez-Palacios, H., 1994. Effect of lipid and (n-3) HUFA levels in microdiets on growth, survival and fatty acid composition of larval gilthead seabream (*Sparus auratus*). *Aquaculture*, **124**, 275–282.

Salte, R., Asgard, T. & Liestol, K., 1988. Vitamin E and selenium prophylaxis against 'Hitra disease' in farmed Atlantic salmon – a survival study. *Aquaculture*, **75**, 45–55.

Sanderson, P. & Calder, P.C., 1998. Dietary fish oil diminishes lymphocyte adhesion to macrophage and endothelial cell monolayers. *Immunology*, **94**, 79-87.

Sargent, J.R. & Henderson, R.J., 1993. Marine (n-3) polyunsaturated fatty acids. In *Developments in oil and fats*, (ed. R.J. Hamilton), pp. 32-65. Glasgow: Blackie Academic and Professional.

Satoh, S., Poe, W.E. & Wilson, R.P., 1989. Studies on the essential fatty acid requirement of channel catfish, *Ictalurus punctatus*. *Aquaculture*, **79**, 121–128

Secombes, C.J., 1996. The non-specific immune system: cellular defences. The fish immune system, organism pathogen and environment. *Fish Physiology*, **15**, 63–103.

Secombes, C.J., Clements, K., Ashton, I. & Rowley, A.F., 1994. The effect of eicosanoids on rainbow trout, *Oncorhynchus mykiss*, leucocyte proliferation. *Veterinary Immunology and Immunopathology*, **42**, 367–378.

Sheely, P.J., Morissey, P.A. & Flynn, A., 1993. Influence of heated vegetable oils and alpha-tocopheryl acetate supplementation on alpha-tocopherol, fatty acids and lipid peroxidation in chicken muscle. *British Poultry Science*, **34**, 367–381.

Sheldon, W.M. Jr & Blazer, V.S., 1991. Influence of dietary lipid and temperature on bactericidal activity of channel catfish macrophages. *Journal of Aquatic Animal Health*, **3**, 87–93.

Smith, C.E., 1979. The prevention of liver lipoid degeneration (ceroidosis) and microcytic anaemia in rainbow trout *Salmo gairdneri* Richardson fed rancid diets: a preliminary report. *Journal of Fish Diseases*, **2**, 429–437.

Smith, W.L. & Fitzpatrick, F.A., 1996. The eicosanoids: cyclooxygenase, lipoxygenase, and epoxygenase pathways. In *Biochemistry of lipids, lipoproteins and membranes* (ed. D.E. Vance and J.Vance), New Comprehensive Biochemistry, **3**, 283-308. Amsterdam: Elsevier.

Spotte, S., 1992. *Captive seawater fishes: science and technology*. Chichester: John Wiley & Sons.

Stephan, G., 1991. Incidence de la supplémentation en lipides péroxidés et de la carence en vitamine E d'un aliment chez trois espèces marines *Dicentrarchus labrax*, *Scophthalmus maximus*, et *Sparus auratus*: Teneurs hépatiques et musculaires en vitamine E et en

substance réagissant à l'acide thiobarbiturique. Apétit, croissance, mortalité. *Ichthyophysiologica Acta*, **14**, 75–86.

Stephan, G., Messager, J.L., Lamour, F. & Baudin-Laurencin, F., 1993. Interactions between dietary alpha-tocopherol and oxidised oil on sea-bass *Dicentrarchus labrax*. In *Fish nutrition in practice, Biarritz, France, 24–27 June 1991* (ed. INRA), pp. 215–218. Paris: INRA. [Les Colloques, no. 61.]

Strasser, T., Fisher, S. & Weber, P.C., 1985. Leukotriene B_5 is formed in human neutrophiils after dietary supplementation with eicosapentaenoic acid. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 1540–1543.

Stryer, L., 1995. *Biochemistry*, 4th ed. New York: W.H. Freeman & Co.

Sure, B., 1924. Dietary requirements for reproduction. II. The existence of a specific vitamin for reproduction. *Journal of Biological Chemistry*, **58**, 693–709.

Takahashi, M., Tsuchiya, J. & Niki, E., 1989. Mode of action of vitamin E as antioxidant in the membranes as studied by spin labelling. *Annals of the New York Academy of Sciences*, **570**, 521–523.

Takeuchi, T. & Watanabe, T., 1977. Effect of eicosapentaenoic acid and docosahexaenoic acid in pollock liver oil on growth and fatty acid composition of rainbow trout. *Bulletin of the Japanese Society of Scientific Fisheries*, **43** (8), 947–953.

Tanaka, J., Fujiwara, H. & Torisu, M., 1979. Vitamin E and immune response. I. Enhancement of helper T cell activity by dietary supplementation of vitamin E in mice. *Immunology*, **38**, 727–734.

Tappia, P.S. & Grimble, R.F., 1994. Complex modulation of cytokine induction by endotoxin and tumour necrosis factor from peritoneal macrophages of rats by diets containing fats of different saturated, monounsaturated and polyunsaturated fatty acid composition. *Clinical Science*, **87**, 173-178.

Tappia, P.S. & Grimble, R.F., 1996. The relationship between altered membrane composition, eicosanoids and TNF-induced IL1 and IL6 production in macrophages of rats fed fats of different unsaturated fatty acid composition. *Molecular and Cellular Biochemistry*, **165**, 135-143.

Tappia, P.S., Ladha, S., Clark, D.C. & Grimble, R.F., 1997. The influence of membrane fluidity, TNF receptor binding, cAMP production and GTPase activity on macrophage cytokine production in rats fed a variety of fat diets. *Molecular and Cellular Biochemistry*, **166**, 135-143.

Tengerdy, R.P., 1989. Vitamin E, immune response, and disease resistance. *Annals of the New York Academy of Sciences*, **570**, 335–344.

Tengerdy, R.P., 1990. The role of vitamin E in immune response and disease resistance. *Annals of the New York Academy of Sciences*, **587**, 24–33.

Terano, T., Salmon, J.A. & Moncada, S., 1984. Effect of orally administered eicosapentaenoic acid (EPA) on the formation of leukotriene B_4 and leukotriene B_5 by rat leukocytes. *Biochemical Pharmacology*, **33** (19), 3071–3076.

Thomassen, M.S. & Røsjø, C., 1989. Different fats in feed for salmon: influence on sensory parameters, growth rate and fatty acids in muscle and heart. *Aquaculture*, **79**, 129–135.

Thompson, K.D., Tatner, M.F. & Henderson, R.J., 1996. Effects of dietary (n-3) and (n-6) polyunsaturated fatty acid ratio on the immune response of Atlantic salmon, *Salmo salar* L. *Aquaculture Nutrition*, **2**, 21–31.

Thorarinsson, R., Landolt, M.L., Elliot, D.G., Pascho, R.J. & Hardy, R.W., 1994. Effect of dietary vitamin E and selenium on growth, survival and the prevalance of *Renibacterium salmoninarum* infection in Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture*, **121**, 343–358.

Tocher, D.R. & Sargent, J.R., 1987. The effect of calcium ionophore A23187 on the metabolism of arachidonic and eicosapentaneoic acids in neutrophils from a marine teleost fish rich in (n-3) polyunsaturated fatty acids. *Comparative Biochemistry and Physiology*, **87B** (4), 733–739.

Ury, H.K., 1976. A comparison of four procedures for multiple comparisons among means (pairwise constrasts) for arbitrary sample sizes. *Technometrics*, **18**, 89-97.

Verlhac, V., N'Doye, A., Gabaudan, J., Troutaud, D. & Deschaux, P., 1991. Vitamin nutrition and fish immunity: influence of antioxidant vitamins (C and E) on immune response of rainbow trout. *In Fish nutrition in practice, Biarritz, France, 24–27 June, 1991* (ed. INRA), Paris: INRA. [Les Colloques, no. 61.]

Verlhac, V. & Gabaudan, J., 1997. Influence of vitamin E on the non specific and specific immune resonse of rainbow trout (*Oncorhynchus mykiss*). *World Aquaculture 97*, February 19-23, Seattle, WA, USA.

Waagbø, R., Sandnes, K., Espield, S. & Lie, Ø., 1988. Haematological and biochemical analyses of Atlantic salmon, *Salmo salar* L., suffering from coldwater vibriosis ('Hitra disease'). *Journal of Fish Disease*, **11**, 417–423.

Waagbø, R., Sandnes, K., Lie, Ø. & Nilsen, E.R., 1993a. Health aspects of dietary lipid sources and vitamin E in Atlantic salmon (*Salmo salar*). I. Erythrocyte total lipid fatty acid composition, haematology and humoral immune response. *Fiskeridirektoratets Skrifter, Serie Ernaering*, **6**(1), 47–62.

Waagbø, R., Sandnes, K., Jørgensen, J., Engstad, R., Glette, J. & Lie, Ø., 1993b. Health aspects of dietary lipid sources and vitamin E in Atlantic salmon (*Salmo salar*). II. Spleen and erythrocyte phospholipid fatty acid composition, nonspecific immunity and disease resistance. *Fiskeridirektoratets Skrifter, Serie Ernaering*, **6**(1), 63–80.

Waagbø, R., Sandnes, K., Sandvin, A. & Lie, Ø., 1991. Feeding three levels of (n-3) polyunsaturated fatty acids at two levels of vitamin E to Atlantic salmon (*Salmo salar*). Growth and chemical composition. *Fiskeridirektoratets Skrifter, Serie Ernaering*, **4**(1), 51–63.

Wang, Y., Huang, D.S., Eskelson, C.D. & Watson, R.R., 1994. Long-term dietary vitamin E retards development of retrovirus induced disregulation in cytokine production. *Clinical Immunology and Immunopathology*, **72(1)**, 70-75.

Wasserman, K., Lovell Becker, E. & Fishman, A.P., 1953. Transport of phenol red in the flounder renal tubule. *Journal of Cellular and Comparative Physiology*, **42**, 385–393.

Watanabe, T., 1982. Lipid nutrition in fish. *Comparative Biochemistry and Physiology*, **73B**(1), 3–15.

Watanabe, T. & Hashimoto, Y., 1968. Toxic component of oxidised saury oil inducing muscular distrophy in carp. *Bulletin of the Japanese Society of Scientific Fisheries*, **34**(12), 1131-1140.

Watanabe, T., Takashima, F. & Ogino, C., 1974. Effect of methyl linolenate on growth of rainbow trout. *Bulletin of the Japanese Society of Scientific Fisheries*, **40**(2), 181–188.

Watanabe, T., Takashima, F., Ogino, C. & Hibiya, T., 1970a. Effects of α-tocopherol deficiency on carp. *Bulletin of the Japanese Society of Scientific Fisheries*, **36**(6), 623–630.

Watanabe, T., Takashima, F., Ogino, C. & Hibiya, T., 1970b. Requirements of young carp for α-tocopherol. *Bulletin of the Japanese Society of Scientific Fisheries*, **36**(9), 972–976.

Watanabe, T., Utsue, O., Kobayashi, I. & Ogino, C., 1975a. Effect of dietary methyl linoleate and linolenate on growth of carp – I. *Bulletin of the Japanese Society of Scientific Fisheries*, **41**(2), 257–262.

Watanabe, T., Takeushi, T. & Ogino, C., 1975b. Effect of dietary methyl linoleate and linolenate on growth of carp – II. *Bulletin of the Japanese Society of Scientific Fisheries*, **41**(2), 263–269.

Watanabe, T., Takeushi, T., Matsui, M., Ogino, C. & Kawabata, T., 1977. Effect of α-tocopherol deficiency on carp. VII. The relationship between dietary levels of linoleate and α-tocopherol requirement. *Bulletin of the Japanese Society of Scientific Fisheries*, **43**(8), 935–946.

Watanabe, T., Takeushi, T., Wada, M. & Uehara, R., 1981a. The relationship between dietary lipid levels and α-tocopherol requirement of rainbow trout. *Bulletin of the Japanese Society of Scientific Fisheries*, **47**(11), 1463–1471.

Watanabe, T., Takeushi, T. & Wada, M., 1981b. Dietary lipid levels and α-tocopherol requirement of carp. *Bulletin of the Japanese Society of Scientific Fisheries*, **47**(12), 1585–1590.

Wise, D.J., Tomasso, J.R., Gatlin III, D.M., Bai, S.C. & Blazer, V.S., 1993a. Effects of dietary selenium and vitamin E on red blood cell peroxidation, gluthatione peroxidase activity, and macrophage superoxide anion production in channel catfish. *Journal of Aquatic Animal Health*, **5**, 177–182.

Wise D.J., Tomasso, J.R., Schwedler, T.E., Blazer, V.S. & Gatlin III, D.M., 1993b. Effect of vitamin E on the immune response of channel catfish to *Edwarselia ictaluri*. *Journal of Aquatic Animal Health*, **5**, 183–188.

Woodall, A.N., Ashley, L.M., Halver, J.E., Olcott, H.S. & Veen, J. van der, 1964. Nutrition of salmonids fishes. XIII. The α-tocopherol requirement of Chinook salmon. *Journal of Nutrition*, **84**, 125–135.

Wu, D., Mura, C., Beharka, A.A., Han, S.H., Paulson, K.E., Hwang, D. & Meydani, S.N., 1998. Age-associated increase in PGE_2 synthesis and COX activity in murine macrophages is reversed by vitamin E. *American Journal of Physiology*, **275**, C661-C668.

Yamashita, H., 1967. Haematological study of a species of rockfish. II. Changes of the moisture content of blood, specific gravity, serum protein, haematocrit value and urea nitrogen level

of serum in the specimens affected by ulcers. *Bulletin of the Japanese Society of Scientific Fisheries*, **33**(11), 995–1002.

Yano, T., 1996. The non-specific immune system: humoral defences. The fish immune system, organism pathogen and environment. *Fish Physiology*, **15**, 106–157.

Yaqoob, P., Newsholme, E.A. & Calder, P.C., 1994. Inhibition of natural killer cell activity by dietary lipids. *Immunology Letters*, **41**, 241–247.

Yu, B.P., 1994. Cellular defences against damage from reactive oxygen species. *Physiological Reviews*, **7**(1), 139–162.

Zapata, A.G., Chiba, A. & Varas, A., 1996. Cells and tissues of the immune system of fish. The fish immune system, organism pathogen and environment. *Fish Physiology*, **15**, 1–62.

Zuasti, A. & Ferrer, C., 1989. Haemopoiesis in the head-kidney of *Sparus auratus*. *Archives of Histology and Cytology*, **52**(3), 249-255.